UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA

CHARACTERISTICS OF PNEUMOCOCCI CAUSING DISEASE IN ADULTS IN PORTUGAL IN A TIME OF PRIVATE USE OF PNEUMOCOCCAL CONJUGATE VACCINES IN CHILDREN

ANDREIA DAS NEVES HORÁCIO

Orientador: Professor Doutor José Augusto Gamito Melo Cristino

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências e Tecnologias da Saúde, Ramo de Microbiologia

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Aos meus pais.

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SUMMARY

Invasive pneumococcal disease (IPD) and non-invasive pneumococcal pneumonia (NIPP) are important causes of morbidity and mortality worldwide, particularly among young children, the elderly and the immunocompromised. Two types of vaccines are available to prevent pneumococcal disease, but these target a limited number of the 97 pneumococcal serotypes described so far. One type is a strictly polysaccharide-based vaccine, which includes 23 serotypes and is primarily indicated for adults (23-valent pneumococcal polysaccharide vaccine, PPV23, targeting serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F). The other type are pneumococcal conjugate vaccines (PCVs). Three PCVs have been licensed to date: a 7-valent formulation (PCV7), which covers serotypes 4, 6B, 9V, 14, 18C, 19F and 23F; a 10-formulation (PCV10), which includes all serotypes of PCV7, plus serotypes 1, 5 and 7F; and a 13-valent formulation (PCV13), which targets all serotypes of PCV10 and also serotypes 3, 6A and 19A.

PCV7 became available in the USA in 2000 and in Europe in 2001. Several studies reported that the use of PCV7 in children was followed by decreases in the incidence of PCV7-type IPD in children. Since PCV7 reduced colonization due to the vaccine serotypes and because children are the main reservoirs of pneumococci in the community, PCV7 serotypes became less transmitted from children to the remaining population, with this resulting in decreases in the incidence of PCV7-type IPD also in non-vaccinated people, including adults (herd protection). However, at the same time, increases in the incidence of IPD due to particular non-PCV7 serotypes occurred in children and adults.

In Portugal, PCV7 was used in children between 2001 and 2009, but was not included in the national immunization program. The uptake of PCV7 was initially low (43% in 2004), but increased steadily (75% in 2008). In mid-2009 and early-2010, PCV10 and PCV13, respectively, became available for children, with PCV13 replacing PCV7. PCV10 and PCV13 were given through the private market until June 2015, when PCV13 was included in the national immunization program for children. During the availability of PCV10 and PCV13 outside the national immunization program, PCV13

was the most frequently used PCV. Even though PPV23 and PCV13 are also available for adults, in Portugal, adult pneumococcal vaccination is estimated to be low.

The studies presented in this thesis aimed to evaluate the characteristics of pneumococci causing adult IPD and adult NIPP in Portugal in a time of private PCVs use in children in the country. The isolates were collected by an epidemiological surveillance network, in work since 1999, which includes several laboratories throughout the country. All isolates analyzed were collected from adult patients (≥ 18 yrs).

For the characterization of adult IPD isolates we determined the serotype and antimicrobial susceptibility of isolates responsible for adult IPD between 2009 and 2014 (n = 2428). The results were compared with previously published data from the same network (1999-2008, n = 2182). Adult IPD isolates were also characterized by Multi Locus Sequence Typing (MLST) and regarding the presence and type of two pilus islands (PI-1 and PI-2). For this characterization, 50% of adult IPD isolates recovered from 2008 to 2011 (n = 871), from each serotype, were randomly chosen. For the characterization of adult NIPP isolates, we determine the serotype and antimicrobial susceptibility of a collection of isolates responsible for adult NIPP between 1999 and 2015 (n = 2735).

Previous studies from this epidemiological surveillance network, which analyzed the serotypes of adult IPD isolates recovered between 1999 and 2008, found that in Portugal there was a significant decrease in the proportion of PCV7 serotypes in adult IPD in the post-PCV7 period (from 30.3% in 1999-2003 to 16.4% in 2008, $p < 0.001$), accompanied by increases in the proportion of specific non-PCV7 serotypes (serotypes 1, 7F and 19A). When analyzing adult IPD data from 2009 to 2014, we found further changes in the serotype distribution of pneumococci causing adult IPD. Comparing adult IPD isolates recovered in 2009-2011 with those recovered in 2012-2014, a new but small decrease in the representation of PCV7 serotypes in adult IPD was noted (from 19% to 14%, $p = 0.003$). In what concerns the overall proportion of PCV13 serotypes, it peaked in 2008 (70%) and then started a significant and gradual decline until 2014 (38%, $p < 0.001$), the last year analyzed. Since PCV10 and PCV13 became available in Portugal only in mid-2009 and early-2010, respectively, the initial decline in the overall proportion of PCV13 serotypes was independent of childhood

vaccination. The PCV13 serotypes that decreased the most from 2008 to 2011 were serotypes 1 (from 10.7% in 2009 to 4.1% in 2011, $p < 0.001$) and 5 (from 2.0% in 2008 to 0% in 2011, $p = 0.003$). The early decreases of these two serotypes may be associated with long term fluctuations and outbreaks, described elsewhere. Other serotypes, instead, decreased when a herd effect with the use of PCV13 in children was expected. This was the case of serotype 7F (from 8.2% in 2012 to 2.7% in 2014, $p < 0.001$) and 19A (from 9.7% in 2012 to 5.6% in 2014, $p = 0.027$). In the post-PCV13 period, there were also significant increases in some of the serotypes not covered by PCV13 (i.e. serotypes 8, 15A, 20 and 22F). In what concerns antimicrobial resistance, and considering current Clinical Laboratory Standards Institute (CLSI) breakpoints, in 2012-2014, 0.5% of the isolates were considered penicillin non-susceptible pneumococci (PNSP) and 17% erythromycin resistant pneumococci (ERP).

Regarding the characterization of adult IPD isolates by MLST we found high genetic diversity, with 206 different sequence types (STs) detected. The STs represented 80 different clonal complexes (CCs), but the six more frequent CCs accounted for half of the isolates (CC156, CC191, CC180, CC306, CC62 and CC230). Most of the STs detected related to STs described in other countries. We found the changes in serotypes occurring in adult IPD following PCV7 use in children were mostly driven by the expansion of previously circulating clones or to decreases in most of the lineages expressing a given serotype. Concerning the presence and type of PI-1 and PI-2, only a small proportion of isolates was positive for any of the PIs (31.9%). Most of the isolates expressing PCV7 serotypes presented PI-1 (87.9%), while PI-2 positive isolates were mainly found among isolates expressing serotypes 1 and 7F, which are serotypes included in PCV10 and PCV13.

In what concerns the characterization of adult NIPP isolates, we found significant declines in the proportion of vaccines serotypes following the use of PCVs in children, although these declines were less marked than those occurring in adult IPD. In adult NIPP, the proportion of PCV7 serotypes declined from 31% in 1999-2003 to 11% in 2011 ($p < 0.001$), while the overall proportion of PCV13 serotypes declined from 44% in 2010 to 30% in 2015 ($p < 0.001$). When considering the 2012-2015 period, and according to current CLSI breakpoints, 1% of adult NIPP isolates were PNSP and

21.7% were ERP. Comparison of NIPP serotypes with IPD serotypes identified associations of several serotypes with either disease presentation.

The studies presented in this thesis showed that in Portugal in the time of PCVs use in children outside the national immunization program there were several changes in the characteristics of pneumococci causing disease in adults. While some of the changes suggested herd protection with the use of PCVs in children, others were independent. The inclusion of PCV13 in the national immunization program for children in June 2015 may be further reducing the importance of PCV13 serotypes in adult IPD and adult NIPP. However, increases of particular non-PCV13 serotypes in adult IPD are concerning and should be monitored. Continued IPD and NIPP epidemiological surveillance is important due to different serotype distribution and dynamics of pneumococci causing each disease presentation.

Key-words: *Streptococcus pneumoniae*; pneumococcal disease in adults; pneumococcal conjugate vaccines; herd protection.

RESUMO

Streptococcus pneumoniae (pneumococo) é um agente bacteriano colonizador da nasofaringe humana, sobretudo das crianças mais jovens, mas que tem também a capacidade de causar infeção em indivíduos de todas as idades. Comparando com a frequência de colonização, a infeção pneumocócica pode ser considerada rara. Ainda assim, as infeções pneumocócicas são das infeções bacterianas mais comuns em todo o mundo. Alguns indivíduos, como as crianças mais jovens e os adultos mais velhos, são particularmente suscetíveis ao desenvolvimento de infeção por pneumococos. As infeções pneumocócicas invasivas e a pneumonia pneumocócica não bacteriémica causam elevada morbilidade e mortalidade em todo o mundo, ocorrendo mortes por estas infeções mesmo nos países onde existem antibióticos eficazes no seu tratamento.

A maioria das estirpes[1] de pneumococos apresenta uma cápsula polissacarídica. Os polissacáridos capsulares são diversos e com base nessa diversidade definem-se serotipos em pneumococos, tendo já sido descritos 97 serotipos. Estima-se que todos os serotipos de pneumococos sejam capazes de causar doença no homem, mas alguns fazem-no muito mais frequentemente que os outros. As vacinas disponíveis para prevenir a infeção pneumocócica baseiam-se num número limitado de serotipos, escolhidos por serem frequentes em infeção invasiva. A vacina Pn23 é exclusivamente polissacarídica, cobre 23 serotipos de pneumococos (serotipos 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F e 33F) e está indicada sobretudo para adultos. Não é usada em crianças menores de dois anos, por não ser imunogénica neste grupo etário. As outras vacinas pneumocócicas em uso têm os polissacáridos capsulares acoplados a proteínas transportadoras adjuvantes e já são indicadas para crianças menores de dois anos. Até ao momento, já se licenciaram três vacinas deste tipo: a vacina Pn7, que cobre sete serotipos de pneumococos (4, 6B, 9V, 14, 18C, 19F e 23F), a vacina Pn10, que cobre os serotipos da vacina Pn7 e ainda os serotipos 1, 5 e 7F, e a vacina Pn13, que cobre os serotipos da vacina Pn10 e ainda os serotipos 3, 6A e 19A.

[1] Neste trabalho, a palavra estirpe é utilizada para referir um microrganismo isolado de um determinado produto biológico.

A vacina Pn7 foi a primeira vacina pneumocócica conjugada a ser comercializada, tendo sido licenciada nos Estados Unidos da América no ano de 2000 e na Europa em 2001. Estudos em vários países reportaram que o uso da vacina Pn7 em crianças foi acompanhado por uma diminuição na incidência de infeção invasiva, pelos serotipos vacinais, em crianças. Como ocorreu redução da colonização nasofaríngea pelos serotipos da vacina Pn7 nas crianças vacinadas, menos destes serotipos foram transmitidos das crianças para a restante comunidade, e menor foi a incidência de infeção invasiva por estes serotipos nos indivíduos não vacinados, incluindo nos adultos (imunidade de grupo). Contudo, ocorreram também aumentos na incidência de infeção invasiva por serotipos não cobertos pela vacina Pn7, em crianças e adultos.

Em Portugal, a vacina Pn7 foi usada em crianças entre 2001 e 2009, mas não fez parte do plano nacional de vacinação (PNV). A cobertura vacinal foi inicialmente baixa (ex. 43% em 2004), mas aumentou progressivamente (ex. 75% em 2008). No final de 2009 e no início de 2010 disponibilizaram-se as vacinas Pn10 e Pn13, respetivamente, sendo que a vacina Pn13 veio substituir a vacina Pn7. As vacinas Pn10 e Pn13 foram usadas fora do PNV até Junho de 2015, altura em que a vacina Pn13 integrou o PNV das crianças. Durante o período de uso das vacinas Pn10 e Pn13 fora do PNV, a vacina mais usada foi a vacina Pn13. Embora as vacinas pneumocócicas também estejam disponíveis para adultos em Portugal (Pn23, desde 1996 e Pn13, desde 2012), estima-se que a grande maioria dos adultos não as receba.

Os estudos apresentados nesta tese tiveram como objetivo avaliar as características dos pneumococos responsáveis por infeções invasivas ou por pneumonia não bacteriémica em indivíduos adultos (≥ 18 anos) em Portugal, num contexto de uso das vacinas pneumocócicas conjugadas em crianças, fora do PNV. As estirpes analisadas nestes estudos foram obtidas através de um sistema de vigilância epidemiológica, em curso desde 1999, e que integra vários laboratórios distribuídos por todo o país.

Para a caracterização das estirpes responsáveis por infeção invasiva em adultos determinou-se o serotipo e a suscetibilidade aos antimicrobianos de todas as estirpes invasivas recolhidas de adultos entre os anos de 2009 e 2014 (n =2428). Os resultados obtidos foram comparados com resultados anteriores (1999-2008, n =2182), da mesma

rede de vigilância epidemiológica. As estirpes responsáveis por infeção invasiva em adultos foram ainda caracterizadas quanto à sua estrutura clonal, através da técnica de *Multi Locus Sequence Typing* (MLST), e quanto à presença e distribuição de duas ilhas genéticas (PI-1 e PI-2), envolvidas na codificação de estruturas do tipo *pilus*. Para esta caracterização escolheu-se, de forma aleatória e para cada serotipo, pelo menos 50% das estirpes isoladas entre os anos de 2008 e 2011 (n =871). No que respeita à caracterização das estirpes isoladas de adultos com pneumonia não bacteriémica determinou-se o serotipo e a suscetibilidade aos antimicrobianos de uma coleção de estirpes isoladas entre os anos de 1999 e 2015 (n =2735).

Em estudos prévios deste sistema de vigilância epidemiológica, em que se analisaram as estirpes isoladas de adultos com infeção invasiva entre os anos de 1999 e 2008, reportou-se que, mesmo numa situação de baixa a moderada cobertura vacinal em crianças com a vacina Pn7, ocorreu uma diminuição significativa na proporção dos serotipos da vacina Pn7 (de 30,3% em 1999-2003 para 16,4% em 2008, p <0.001), acompanhada por um aumento significativo na proporção de alguns serotipos não incluídos nesta vacina (serotipos 1, 7F e 19A). Neste trabalho, com a análise das estirpes isoladas de infeção invasiva em adultos entre os anos de 2009 e 2014, verificou-se que continuaram a ocorrer alterações significativas na importância relativa dos diferentes serotipos. Quando comparando as estirpes de pneumococos isoladas em 2009-2011 com as isoladas em 2012-2014 detetou-se uma nova diminuição na representação dos serotipos da vacina Pn7 (de 19% para 14%, p =0,003). Quanto aos serotipos da vacina Pn13, que no seu conjunto causaram 70% das infeções pneumocócicas invasivas em adultos em 2008, verificou-se que iniciaram uma descida gradual na sua proporção, até 38% em 2014 (p <0,001), o último ano estudado para as infeções invasivas. Os serotipos 1 (diminuiu de 10,7% em 2008 para 4,1% em 2011, p <0,001) e 5 (diminuiu de 2,0% em 2008 para 0% em 2011, p = 0,003) foram os que mais contribuíram para a descida inicial na proporção geral dos serotipos da vacina Pn13. A diminuição destes serotipos ocorreu antes de se esperar um efeito de imunidade de grupo pelo uso das vacinas Pn10/Pn13 em crianças e poderá estar relacionada com características intrínsecas destes serotipos, nomeadamente, flutuações prolongadas de prevalência e capacidade para causar surtos. Os serotipos 7F (diminuiu de 8,2% em 2012 para 2,7% em 2014, p <0,001) e 19A (diminuiu de 9,7% em 2012 para 5,6% em

2014, p =0,027) foram os que mais contribuíram para a diminuição geral na proporção dos serotipos da vacina Pn13, no período em que já se esperava um efeito de imunidade de grupo pelo uso da vacina Pn13 em crianças. Entre os anos de 2008 e 2014 ocorreram também aumentos importantes e significativos em alguns dos serotipos não cobertos pela vacina Pn13 (serotipos 8, 15A, 20 e 22F). Considerando as normas do *Clinical Laboratory Standards Institute* (CLSI), disponibilizadas em 2008 e ainda em vigor, a não suscetibilidade à penicilina foi rara entre as estirpes invasivas não responsáveis por meningite (0,5% de não suscetibilidade à penicilina em 2012-2014), mas considerável entre as estirpes responsáveis por meningite (20,3% de resistência em 2012-2014). A resistência à eritromicina, que tinha aumentado durante o período de uso da vacina Pn7 em crianças, diminuiu entre os anos de 2010 e 2014 (de 24,8% para 15,7%, p =0,005).

Quanto à caracterização por MLST das estirpes isoladas de adultos com infeção invasiva detetou-se uma elevada diversidade genética, com 206 perfis alélicos (STs), organizados em 80 complexos clonais (CCs). Contudo, metade das estirpes distribuiu-se por apenas 6 CCs (CC156, CC191, CC180, CC306, CC62 e CC230). Muitos dos STs encontrados relacionaram-se com STs descritos noutros países. Verificou-se que as mudanças na distribuição de serotipos que ocorreram no período pós-Pn7 deveram-se à expansão de linhagens genéticas previamente existentes, ou à diminuição da maioria das linhagens genéticas associadas a um dado serotipo. Relativamente à presença e distribuição das ilhas PI-1 e PI-2 verificou-se que apenas 31,9% das estirpes apresentavam pelo menos uma das ilhas genéticas. A maioria dos serotipos cobertos pela vacina Pn7 apresentaram a ilha PI-1 (87,9%), enquanto a ilha PI-2 surgiu sobretudo entre as estirpes de serotipo 1 e 7F, que são serotipos incluídos nas vacinas Pn10 e Pn13.

Relativamente à caracterização dos pneumococos isolados de adultos com pneumonia não bacteriémica detetaram-se mudanças significativas na distribuição de serotipos durante o uso das vacinas conjugadas em crianças, embora estas alterações tenham sido menos pronunciadas que aquelas encontradas entre as estirpes causadoras de infeção invasiva. Quanto à representação dos serotipos da vacina Pn7 verificou-se que passou de 31% em 1999-2003 para 11% em 2011 (p <0,001). Relativamente à proporção geral dos serotipos da vacina Pn13, esta diminuiu de 44%

em 2010 para 30% em 2015 (p <0,001). A descida na representação dos serotipos da vacina Pn13 foi principalmente provocada por descidas pouco acentuadas na proporção relativa dos serotipos 3 e 19A. Tendo em conta as normas de CLSI atuais, no período de 2012-2015, 1,0% das estirpes apresentaram não suscetibilidade à penicilina e 21,7% das estirpes apresentaram resistência à eritromicina. Comparando a distribuição de serotipos das estirpes isoladas de adultos com pneumonia não bacteriémica com as isoladas de adultos com infeção invasiva verificou-se uma distribuição de serotipos própria, com vários serotipos a estarem significativamente mais associados a um ou outro tipo de infeção.

Os trabalhos apresentados nesta tese mostraram que após a disponibilização e uso das vacinas pneumocócicas conjugadas em crianças, fora do PNV, ocorreram diversas alterações na distribuição de serotipos de pneumococos responsáveis por infeções em adultos em Portugal. Algumas das alterações detetadas sugeriram um efeito de imunidade de grupo, enquanto outras foram independentes. Com a introdução, em Junho de 2015, da vacina Pn13 no PNV das crianças espera-se uma representação cada vez menor dos serotipos vacinais entre os pneumococos responsáveis por infeções em adultos. Contudo, o aumento significativo de alguns dos serotipos não cobertos pela vacina Pn13 é preocupante e deve ser monitorizado. Dada a diferente distribuição de serotipos em infeção invasiva e em pneumonia não bacteriémica torna-se importante continuar a vigilância epidemiológica de ambas as populações pneumocócicas.

Palavras-chave: *Streptococcus pneumoniae*; infeção pneumocócica em adultos; vacinas pneumocócicas conjugadas; imunidade de grupo.

THESIS OUTLINE

This thesis is composed of five chapters. Chapter I corresponds to a general introduction focusing on *Streptococcus pneumoniae*, the human-pneumococcal interaction and the available strategies to treat and prevent pneumococcal infections. At the end of this chapter the aims of the thesis are described. Chapter II, III and IV are composed of full reproductions of the publications produced during the PhD work. A rationale is provided at the beginning of chapters II, III and IV. Chapter II is composed of two publications evaluating the serotype and antimicrobial susceptibility of pneumococcal isolates causing invasive disease in adults in Portugal in two different periods (2009-2011 and 2012-2014, respectively). Chapter III is composed of one publication that studies the clonal structure of pneumococcal isolates causing invasive disease in adults in Portugal in 2008-2011. Chapter IV presents two manuscripts that study the serotype and antimicrobial susceptibility of pneumococcal isolates causing non-invasive pneumonia in adults in Portugal in two different periods (1999-2011 and 2012-2015, respectively). Chapter V corresponds to a general discussion where the main findings obtained in the studies of the previous chapters are approached and discussed. Concluding remarks and future perspectives are presented at the end of this chapter. All references used throughout the thesis are presented together in a section called "References". Facsimiles of all published manuscripts are presented in the last section of this thesis, which was named "Publications".

The studies presented in this thesis are reproductions of the following publications:

Horácio, A. N., Diamantino-Miranda, J., Aguiar, S. I., Ramirez, M., Melo-Cristino, J., and the Portuguese Group for the Study of Streptococcal Infections (2013). The majority of adult pneumococcal invasive infections in Portugal are still potentially vaccine preventable in spite of significant declines of serotypes 1 and 5. *PLoS One* 8, e73704.

Horácio, A. N., Silva-Costa, C., Lopes, J.P., Ramirez, M., Melo-Cristino, J., and the Portuguese Group for the Study of Streptococcal Infections (2016). Serotype 3 remains the leading cause of invasive pneumococcal disease in adults in Portugal (2012–2014) despite continued reductions in other 13-valent conjugate vaccine serotypes. *Front. Microbiol.* 7, 1616.

Horácio, A. N., Silva-Costa, C., Diamantino-Miranda, J., Lopes, J. P., Ramirez, M., Melo-Cristino, J., et al. (2016). Population structure of *Streptococcus pneumoniae* causing invasive disease in adults in Portugal before PCV13 availability for adults: 2008-2011. *PLoS One* 11, e0153602.

Horácio, A. N., Lopes, J. P., Ramirez, M., Melo-Cristino, J., and the Portuguese Group for the Study of Streptococcal Infections (2014). Non-invasive pneumococcal pneumonia in Portugal – serotype distribution and antimicrobial resistance. *PLoS One* 9, e103092.

Horácio, A. N., Silva-Costa, C., Lopes, E., Ramirez, M., Melo-Cristino, J., on behalf of the Portuguese Group for the Study of Streptococcal Infections (2018). Conjugate vaccine serotypes persist as major causes of non-invasive pneumococcal pneumonia in Portugal despite declines in serotypes 3 and 19A (2012-2015). *PLoS One* 13, e0206912.

LIST OF ABBREVIATIONS

ACIP	Advisory Committee on Immunization Practices
AOM	Acute otitis media
AW	Adjusted Wallace
CAP	Community acquired pneumonia
CAPiTA	Community-Acquired Pneumonia immunization Trial in Adults
CI	Confidence interval
CLSI	Clinical Laboratory Standards Institute
CRM197	Cross-reactive material 197 (non-toxic mutant of diphtheria toxin)
CPS	Capsular polysaccharide
CSF	Cerebral spinal fluid
DLV	Double-locus-variant
EPNSP	Erythromycin and penicillin non-susceptible pneumococci
ERP	Erythromycin resistant pneumococci
FDR	False discovery rate
ICD-10	International Statistical Classification of Diseases and Related Health Problems 10th Revision
IPD	Invasive pneumococcal disease
M	Macrolide resistant phenotype
MIC	Minimum inhibitory concentration
MLEE	Multilocus enzyme electrophoresis
MLS_B	Resistance to macrolides, lincosamides and streptogramins B
MLST	Multi-locus sequence typing
MLVA	Multilocus variable number of tandem repeat analysis
NIP	National immunization program
NIPP	Non-invasive pneumococcal pneumonia
NVT	Non-vaccine type
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction

PCV	Pneumococcal conjugate vaccine
PCV7	7-valent pneumococcal conjugate vaccine
PCV10	10-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PFGE	Pulsed-field gel electrophoresis
PI	Pilus islet
PI-1	Pilus islet 1
PI-2	Pilus islet 2
PMEN	Pneumococcal Molecular Epidemiology Network
PNSP	Penicillin non-susceptible pneumococci
PPV	Pneumococcal polysaccharide vaccine
PPV23	23-valent pneumococcal polysaccharide vaccine
SID	Simpson's index of diversity
SLV	Single-locus-variant
ST	Sequence type
UAD	Urinary antigen detection assay
UPGMA	Unweighted pair group method with arithmetic means
WGS	Whole-genome sequencing
WHO	World Health Organization

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..... **vii**

SUMMARY ..... **ix**

RESUMO ..... **xiii**

THESIS OUTLINE ..... **xix**

LIST OF ABBREVIATIONS ..... **xxi**

TABLE OF CONTENTS ..... **xxiii**

CHAPTER I: GENERAL INTRODUCTION **1**

Early History of *Streptococcus pneumoniae* 3
General Features and Microbiological Identification of *S. pneumoniae* 3
Pneumococcal Infection 5
Pneumococcal Virulence Factors 9
Pneumococcal Typing 11
Antimicrobial Therapy and Resistance in *S. pneumoniae* 16
Pneumococcal Vaccines 20

AIMS OF THE THESIS 33

CHAPTER II: INVASIVE PNEUMOCOCCAL DISEASE IN ADULTS IN PORTUGAL – SEROTYPES AND ANTIMICROBIAL SUSCEPTIBILITY **35**

RATIONALE 37

THE MAJORITY OF ADULT PNEUMOCOCCAL INVASIVE INFECTIONS IN PORTUGAL ARE STILL POTENTIALLY VACCINE PREVENTABLE IN SPITE OF SIGNIFICANT DECLINES OF SEROTYPES 1 AND 5 41

Abstract 41
Introduction 43
Materials and Methods 46
Results 49
Discussion 57
Acknowledgements 63
Supporting Information 65

SEROTYPE 3 REMAINS THE LEADING CAUSE OF INVASIVE PNEUMOCOCCAL DISEASE IN ADULTS IN PORTUGAL (2012–2014) DESPITE CONTINUED REDUCTIONS IN OTHER 13-VALENT CONJUGATE VACCINE SEROTYPES 69

Abstract 69
Introduction 71
Materials and Methods 73
Results 75
Discussion 85
Acknowledgements 89
Supporting Information 91

CHAPTER III: INVASIVE PNEUMOCOCCAL DISEASE – CLONES AND PILI **93**

RATIONALE 95

POPULATION STRUCTURE OF *STREPTOCOCCUS PNEUMONIAE* CAUSING INVASIVE DISEASE IN ADULTS IN PORTUGAL BEFORE PCV13 AVAILABILITY FOR ADULTS: 2008-2011 .. 99

Abstract 99
Introduction 101
Materials and Methods 103
Results 106
Discussion 115
Acknowledgements 121
Supporting Information 123

CHAPTER IV: NON-INVASIVE PNEUMOCOCCAL PNEUMONIA – SEROTYPES AND ANTIMICROBIAL SUSCEPTIBILITY 127

RATIONALE 129

NON-INVASIVE PNEUMOCOCCAL PNEUMONIA IN PORTUGAL — SEROTYPE DISTRIBUTION AND ANTIMICROBIAL RESISTANCE 133

Abstract 133
Introduction 135
Materials and Methods 137
Results 140
Discussion 146
Acknowledgements 152
Supporting Information 154

CONJUGATE VACCINE SEROTYPES PERSIST AS MAJOR CAUSES OF NON-INVASIVE PNEUMOCOCCAL PNEUMONIA IN PORTUGAL DESPITE DECLINES IN SEROTYPES 3 AND 19A (2012-2015) 161

Abstract 161
Introduction 163
Materials and Methods 165
Results 168
Discussion 174
Acknowledgements 179
Supporting Information 181

CHAPTER V – GENERAL DISCUSSION 189

Invasive Pneumococcal Disease 192
Non-invasive Pneumococcal Pneumonia 206
Concluding Remarks and Future Perspectives 212
Supporting Information 219

REFERENCES 225

PUBLICATIONS 251

CHAPTER I: GENERAL INTRODUCTION

Early History of *Streptococcus pneumoniae*

The first observation of *S. pneumoniae* (pneumococcus) is attributed to Klebs, which in 1875 saw cocci in the lung fluid of a men dying with pneumonia. Later, in 1880, Ebert and Mátray may have made independent observations of pneumococci. Ebert, who examined the biological samples of a patient with pneumonia and meningitis, described bacteria consistent with pneumococci. However, probably ignoring the novelty of his observation, he misclassified pneumococci as other species known at that time. Mátray described cocci in the sputum of a patient with pneumonia and named the microorganism "Pneumoniekokken". The first two reports of animal passage and isolation of pneumococci date to 1881. These reports were made, independently, by Pasteur and Sternberg and were based on the inoculation of human saliva in rabbits. Associations of pneumococci with several human diseases occurred during the following decade and were made by several scientists, including Friedländer and Fraenkel (White et al., 1938).

The name "pneumococcus" was given by Fraenkel in 1886 and has been widely used since then. Several other names were attributed to this species, with "*Diplococcus pneumoniae*" being the most frequently used in most part of the 20th century. Pneumococcus was reclassified into the streptococcal genus in 1974 gaining its current name, "*Streptococcus pneumoniae"* (Watson et al., 1993).

The historical importance of *S. pneumoniae* went beyond its implications on disease, as this microorganism was involved in some of the most important scientific discoveries of the past century (Austrian, 1981). One example is the identification of DNA as the hereditary material by Avery, MacLeod and McCarty in 1944 (Avery et al., 1944).

General Features and Microbiological Identification of *S. pneumoniae*

Pneumococci are Gram positive, catalase-negative, facultative anaerobes. Based on its 16S rRNA sequence, *S. pneumoniae* is included in the mitis phylogenetic group of the viridans streptococcus group (Kawamura et al., 1995; Kilian et al., 2008). The more closely related species to *S. pneumoniae* are *S. pseudopneumoniae* and *S. mitis* (Arbique et al., 2004; Yahiaoui et al., 2016). Distinct properties of pneumococcal cells

are their lancet-shaped appearance and their propensity to occur in pairs. Less often, pneumococcal cells group into small chains. This microbe grows optimally at 35-37 ºC and in an atmosphere of 5% CO_2 in air. Pneumococcus usually has a polysaccharide capsule, and this structure is thought to be its major virulence factor. There are several types of capsular polysaccharides (CPS) in pneumococci, each producing a distinct immunological reaction. This forms the basis for pneumococcal serotype classification. So far, based on the different capsular polysaccharides detected, a total of 97 distinct pneumococcal serotypes have been described (Geno et al., 2017). Some pneumococci do not present a capsule or have it downregulated. Such isolates cannot be serotyped and are called non-typable isolates (NTs). Pneumococcus is naturally transformable, which means it can incorporate exogenous DNA into its chromosome. DNA exchanges may occur within the species or with other streptococci (Kilian et al., 2008).

Microbiological identification of pneumococci is sometimes complicated by the fact that all tests lack 100% specificity and sensitivity. Moreover, closely related species to *S. pneumoniae* may not only resemble pneumococci in the laboratory, but also share its habitat and, more rarely, cause pneumococcal-like diseases (Richter et al., 2008). The classical microbiological approach for the identification of pneumococci from culture relies on the examination of colony morphology on blood agar plates and on two additional tests: optochin susceptibility and bile solubility tests (Lund, 1960; Alonso De Velasco et al., 1995). On blood agar plates, pneumococcal colonies are α-hemolytic, translucent and grayish, with 1-2 mm in diameter. They usually present a central depression after 24-48h incubation, which is due to autolysis. Some serotypes present mucoid colonies (Lund, 1960). *S. pneumoniae* is usually sensitive to optochin (ethylhydrocupreine hydrochloride) and this feature, combined with the observation of typical colonies, is sufficient to identify an isolate as *S. pneumoniae* (Perilla et al., 2003). However, non-encapsulated pneumococci produce atypical colonies (rough, smaller and flat), resembling other closely related species. Moreover, optochin resistance exists (Kontiainen and Sivonen, 1987) and is increasing (Aguiar et al., 2006; Yahiaoui et al., 2016). Therefore, doubtful results should be tested by bile solubility test. Pneumococci are usually soluble in bile or deoxycholate. For bile-insoluble isolates (Pease et al., 1986), an alternative approach is to test the

microorganism with anti-capsular serum that reacts with all pneumococcal capsules (Omni-serum) (Richter et al., 2008). However, again, specificity and sensitivity are not total, since closely related species to *S. pneumoniae* may react with pneumococcal anti-capsular serum (Lee et al., 1984) and non-encapsulated pneumococci will not react (Melchiorre et al., 2012). There are other approaches for the identification of pneumococci from culture, such as molecular tests (Richter et al., 2008), but generally, these are less frequently used.

Pneumococcal Infection

Pneumococcus behaves both as a human pathogen and as a common colonizer of the human respiratory tract. Comparing with the frequency of colonization, pneumococcal disease can be considered a relatively rare event of the human-pneumococcal interaction. However, due to the high prevalence of pneumococcal carriage in children and the fact that children serve as a source of pneumococci for the surrounding community, the global burden of pneumococcal disease turns out to be huge.

Pneumococcal Colonization

Pneumococcal colonization is a transient and highly frequent event. All humans are thought to be colonized at least once in life with *S. pneumoniae* with the highest rates of colonization occurring during early childhood (Bogaert et al., 2004). There are reports of pneumococcal colonization in other animal species such as horses (Burrell et al., 1986) cats and dogs (van der Linden et al., 2009), but nonetheless, humans are considered the reservoir of pneumococci. In the human host, *S. pneumoniae* resides in the upper airway, including nasal passages, oropharynx and nasopharynx. Transmission to a susceptible host occurs through direct contact with respiratory secretions of infected individuals (Kadioglu et al., 2008).

Colonization rates vary with several factors, such as age, socioeconomic conditions and ethnicity (Bogaert et al., 2004). In children, the incidence of colonization is highest in the first two to three years of life (Simell et al., 2012), reaching 70% among children attending day care centers (Rodrigues et al., 2009). After the peak, carriage rates decline gradually, stabilizing in individuals > 5 years old

at ~ 10%. (Almeida et al., 2014; Short and Diavatopoulos, 2015). Pneumococcal colonization rates are higher in developing countries for all age groups. The first onset of colonization generally occurs at 6 months of age, but in low income countries colonization can start earlier (Short and Diavatopoulos, 2015). Crowding conditions, like those found in day care centers or in large families with young children, increase the likelihood of carriage (Bogaert et al., 2004). For this reason, parents and grandparents that are in close contact with infants may become more frequently colonized than non-caregivers. Some ethnical groups, such as native American and African American population, exhibit higher prevalence of pneumococcal carriage (Bogaert et al., 2004). Colonization may last from days to months in young children (Raymond et al., 2000) and this variability is serotype dependent (Valente et al., 2016). Co-colonization by two or more pneumococcal strains may occur, but is less common than colonization with a single pneumococcal strain (Valente et al., 2016).

Pneumococcal Disease

Progression from carriage to disease is sporadic, but outbreaks of disease have been documented in crowded settings with susceptible individuals. When human natural defense mechanisms are circumvented, pneumococci may spread from the nasopharynx to related anatomical sites, such as the sinuses, middle year and lungs. If pneumococci reach the bloodstream, they can spread to less readily accessible sites of the body, such as the brain, joints and peritoneal cavity. If there is a fracture of the skull, pneumococci may spread directly into the central nervous system, but in most cases, meningitis is secondary to hematogenous spread (Goldblatt and O'Brien, 2015).

When the bacterial infection is confined to the mucosal surfaces, the infection is considered "non-invasive" and when there is involvement of normally sterile body sites, such as blood or cerebrospinal fluid (CSF), the infection is called "invasive" (Drijkoningen and Rohde, 2014). Pneumococcal pneumonia can be classified either as an invasive or a non-invasive infection, depending on the presence or absence of bacteria in blood, respectively. The proportion of pneumococcal pneumonia that is bacteremic varies widely among studies. Even so, it is generally accepted that non-invasive pneumococcal pneumonia (NIPP) is more frequent than bacteremic pneumonia and that there are at least three cases of NIPP for every case of bacteremic

pneumonia (Said et al., 2013). In adults, invasive pneumococcal disease (IPD) is mainly represented by bacteremic pneumonia (Drijkoningen and Rohde, 2013).

Acute otitis media (AOM) and sinusitis are the most common diseases caused by *S. pneumoniae*. For example, of the four million pneumococcal disease episodes estimated to have occurred in the USA in 2004, 75% was due to either AOM or sinusitis (Huang et al., 2011). However, in that study, pneumococcal pneumonia was estimated to account for > 90% of pneumococcal-related hospitalizations (401,000/445,000) and > 80% of pneumococcal-associated deaths (19,000/22,000). Importantly, among the estimated 19,000 deaths due to pneumococcal pneumonia, 16,000 were in people ≥ 65 years. In this age group, pneumonia was in fact the most frequent manifestation of pneumococcal disease (even more frequent than sinusitis) (Huang et al., 2011).

The true burden of pneumococcal pneumonia is globally underestimated, and the reasons are numerous. First, microbiological investigation is usually reserved for the more severe cases, which are those requiring hospitalization. Second, even when microbiological investigation is performed, only < 40% will result in a definitive diagnosis, since determining the etiology of pneumonia is, by itself, challenging. Third, the clinical diagnosis is difficult and the clinical presentation of community acquired pneumonia (CAP) overlaps with other lower respiratory tract infections, such as acute bronchitis and acute exacerbations of chronic obstructive lung disease (Goldblatt and O'Brien, 2015).

Given that microbiological diagnosis of IPD is unambiguous, the incidence of IPD is commonly used as an indicator of the overall burden of pneumococcal disease (WHO, 2008). However, it is important to bear in mind that this approach fails to include the major portion of the burden of serious pneumococcal disease, which is represented by NIPP.

The incidence of IPD has been changing continuously since the beginning of the 21st century due to the availability of effective vaccines against *S. pneumoniae* for children (discussed later in this introduction). Before the availability of these vaccines, the incidence of IPD in the USA was > 150 cases per 100,000 population among children < 2 years and almost 60 cases per 100,000 population among adults ≥ 65 years (Robinson et al., 2001). In Western Europe, in a similar period, IPD incidence

was highly variable by country. However, several countries reported IPD incidence among young children to be above 50 cases per 100,000 population (Steens et al., 2013; Harboe et al., 2014; Guevara et al., 2014) and around 30 cases per 100,000 population among older adults (Harboe et al., 2014; Guevara et al., 2014; Lepoutre et al., 2015).

Rates of pneumococcal disease vary with several factors, such as sex, ethnicity and the previous health state of the patient. Concerning sex, males are more often affected by pneumococcal disease than females. Regarding ethnicity, some specific ethnicities present higher incidence of pneumococcal disease (e.g. African Americans and Native American populations), perhaps due to socioeconomic conditions and higher prevalence of underlying risk factors for pneumococcal disease. In temperate climate regions, higher rates of pneumococcal disease occur in colder than in warmer months (Goldblatt and O'Brien, 2015). Numerous medical conditions also increase the risk of pneumococcal infection. Some examples are individuals with a debilitated immune system, such as those with HIV, asplenia, sickle cell disease, hematological cancer and under treatment with immunosuppressive drugs. Individuals with reduced ability to eliminate pneumococci from the airways, such as those with chronic obstructive pulmonary disease or asthma are also in increased risk for the development of pneumococcal disease. People with underlying comorbidities such as chronic kidney, liver or heart disease and individuals with diabetes are more prone to pneumococcal disease. The lifestyle of the patient is also important. For example, smoking and alcohol abuse increase the risk of pneumococcal disease (Kyaw et al., 2005; Goldblatt and O'Brien, 2015; Torres et al., 2015). The age of the patient is also very important, with younger children and the elderly being the most affected by pneumococcal disease. Young children are at increased risk for pneumococcal disease due to their likelihood of being colonized by pneumococci and the lack of a completely developed immune system. The elderly are more likely to have health conditions that increase the risk for pneumococcal disease (Vila-Corcoles et al., 2015). However, old age is itself a risk factor for the development of pneumococcal disease (Regev-Yochay et al., 2017). In addition, previous viral respiratory disease (e.g. influenza infection) also puts individuals more vulnerable for pneumococcal infections (Goldblatt and O'Brien, 2015).

Pneumococcal Virulence Factors

Pneumococci present a vast array of virulence factors, which are important for the different stages of the human-pneumococcal interaction. Examples of virulence factors in pneumococci are the polysaccharide capsule, pili, teichoic acids, IgA1 protease and pneumolysin (Kadioglu et al., 2008). The initial colonization of the nasopharynx is mediated by the binding of pneumococci to epithelial cells through surface protein adhesins. The bacterial adhesins include phosphorylcholine (ChoP), which is a constituent of cell-wall teichoic acids and membrane-bound lipoteichoic acids (Cundell et al., 1995). ChoP mediates bacterial adherence to the receptor for platelet-activating factor (rPAF). The natural ligand for rPAF is the platelet-activating factor (PAF), which also contains ChoP. Therefore, the pneumococcus might mimic PAF to use its receptor, which is extensively distributed on host tissues including the epithelial surface of the nasopharynx (Kadioglu et al., 2008). Subsequent migration of pneumococci to the lower respiratory tract can be prevented if the pneumococcal cells are enveloped in mucus and removed from the airways by the action of ciliated epithelial cells. Pneumococcal cells prevent this envelopment in mucus by producing secretory IgA protease and pneumolysin. Secretory IgA traps bacteria in mucus by binding the bacteria to mucin with the Fc region of the antibody. The bacterial IgA protease prevents this interaction (Wani et al., 1996). Pneumolysin is a cholesterol-activated cytolysin, which binds cholesterol in the host cell membrane creating pores (Tilley et al., 2005). This activity can destroy the ciliated epithelial cells and phagocytic cells. The polysaccharide capsule and pili are the most relevant pneumococcal virulence factors for the context of this thesis and are therefore discussed in more detail below.

Polysaccharide Capsule

The polysaccharide capsule is considered the most important virulence factor in *S. pneumoniae*. It forms the outermost layer of pneumococci (Skov Sørensen et al. 1988) being thus the primary target of host's immune system. A high degree of encapsulation act as a barrier that hides pneumococcal surface structures that otherwise would be recognized by antibodies (Hyams et al., 2010). Immune

components that already identified pneumococcal surface antigens but still need to be recognized by phagocytic cells are also more easily hidden in strongly encapsulated pneumococci (Kadioglu et al., 2008). Additionally, a high degree of encapsulation can protect pneumococci against non-opsonic killing by neutrophils during carriage (Weinberger et al., 2009). The expression of a capsule reduces pneumococcal trapping in neutrophil extracellular traps (Wartha et al., 2007) and neutralizes cationic antimicrobial peptides (Llobet et al. 2008). During colonization, pneumococcal capsule was shown to interfere with mucus-mediated clearance. The heavily charged pneumococcal CPS is electrostatically repulsed by mucopolysaccharides and this may impair clearance by mucociliary flow (Nelson et al., 2007). The expression of a thick capsule may not be needed during colonization due to its inhibitory effect on pneumococcal adherence. Most pneumococcal isolates present phase variation between two forms that can be distinguished by their opaque or transparent colony morphologies (Weiser et al., 1994). A thinner capsule (transparent variant) is more advantageous for colonization, as it facilitates pneumococcal biding to host tissues. Therefore, transparent variants prevail over opaque variants during colonization (Weiser et al., 1994). Oxygen availability, which changes in various tissues, can affect CPS production (Weiser et al., 2001). This may be a possible signal for pneumococci to progress from colonization to disease. Despite the general importance of pneumococcal capsules, the ability of pneumococci to colonize, invade or cause severe disease is highly dependent on the serotype, meaning that different CPS may interact differently with the human host (Weinberger et al., 2009).

Pili

Two types of pilus-like structures were identified in pneumococci – pilus 1 and pilus 2. Pilus 1 was shown to influence pneumococcal adherence to lung epithelial cells (Barocchi et al., 2006; Hemsley et al., 2003). In addition, mouse models of pneumococcal pneumonia and bacteremia proposed a role of pilus 1 in virulence and host inflammatory responses (Barocchi et al., 2006; Hava et al., 2002). Immunization of mice with pilus structural antigens induced protection against lethal challenge by piliated strains. Therefore, pilus 1 not only contribute to pneumococcal adherence and virulence but also stimulate the host inflammatory response. (Gianfaldoni et al.,

2007). Pilus 2 was shown to be involved in adherence of pneumococci to host epithelial cells. However, its attachment to host cells was lower than that observed for PI-1 (Bagnoli et al. 2008).

Pilus 1 is encoded in the *rlrA* pathogenicity islet, also known as pilus islet 1 (PI-1). This islet is 14 kilobases (kb) in size and is flanked by IS1167 elements. It contains seven genes, which are necessary for the formation of pilus 1 on the cell surface of pneumococci: the *rlrA* gene encodes a transcriptional regulator, the *rrgA*, *rrgB* and *rrgC* genes encode the proteins composing the pilus itself and the *srtB*, *srtC* and *srtD* genes encode three sortases (Barocchi et al., 2006). Three variants of PI-1 are known. These variants are called clades I, II and III. With the exception of the *rlrA* gene, all other PI-1 genes show some genetic variability. Amplification of the *rlrA* gene can be used to determine the presence or absence of PI-1 (Moschioni et al., 2008). In the case of *rlrA* negative isolates, DNA fragments of three different sizes can be obtained. The fragments of *rlrA* negative isolates and the clades of *rlrA* positive isolates associate with pneumococcal genotypes and serotypes (Aguiar et al., 2008b).

The PI-2 pathogenicity islet, also known as pilus islet 2, is ~ 7 kb in size and is composed of five genes: *pitA*, *sipA*, *pitB*, *srtG1* and *srtG2*. The *pitB* gene encodes the polymers composing pilus 2, the *sipA* gene encodes a signal peptidase, and the *srtG1* gene encodes a sortase. The distribution of PI-2 also correlates with the genotype and serotype (Bagnoli et al. 2008).

Pneumococcal Typing

In 1897, Banzançon and Griffon realized the diversity of pneumococci while using rabbit anti-pneumococcal serum to agglutinate with different pneumococcal isolates. Shortly after, several methods of serotyping were developed and distinct serotypes started being recognized. The interest in serotyping suffered some fluctuations during the 20th century. Before the World War II, the main goals of serotyping were to develop serum treatment, to study the relationships between pneumococcal isolates and to trace the spread of pneumococcal infections (White et al., 1938). Later, the availability of effective antibiotics for the treatment of pneumococcal disease, namely sulfonamides and penicillin, decreased the need for

serum therapy and, consequently, the attention given to pneumococcal serotyping (Austrian, 1976). Moreover, the relatively low discriminatory power of serotyping was revealed when researchers found that only a limited number of pneumococcal serotypes caused most of infections. A renewed interest in serotyping arose with the emergence of antimicrobial resistant pneumococci and the subsequent search for an effective polysaccharide-based vaccine. Today, serotyping is a key element of surveillance studies evaluating the impact of the pneumococcal vaccines in use and monitoring the remaining disease (Ramirez et al., 2015).

The discovery that only a fraction of isolates expressing certain serotypes were associated with antimicrobial resistance promoted a search for a method that would allow discriminating isolates within serotypes. Genotyping methods were used for that purpose and revealed also superior to serotyping for the detection and monitoring of outbreaks. Pneumococcal genotyping has also been of critical value in evolutionary studies. Pulsed field gel-electrophoresis (PFGE) and multi-locus sequence typing (MLST) were for several years the most frequently adopted genotyping methods and are still in use today. However, great developments in whole genome sequencing (WGS) have been made more recently, making WGS the most promising future of pneumococcal typing (Ramirez et al., 2015).

Serotyping

Of all the techniques developed to assess the diversity of pneumococcal capsules, the one that stood out before, and remains today, as the gold standard of serotyping is the serological method developed by Neufeld in 1902, namely the quellung reaction test. “Quellung” stands for “swelling” in German and this term was applied by Neufeld when he witnessed the rapid development of a swollen appearance in a droplet containing pneumococcal broth culture and agglutinating serum (Austrian, 1976). With his method, Neufeld identified the first two types of pneumococci, which were extended to four types in 1913, by Dochez and Gillespie. Types I and II correspond today to serotypes 1 and 2, type III included all mucoid pneumococci and type IV was an heterogeneous group encompassing all the remaining discovered types (White et al., 1938). Later, the detection of new pneumococcal types continued independently by several investigators in different

countries. By 1929, a total of 32 different pneumococcal types had already been described by Cooper et al. in the USA, interestingly, using different serotyping techniques than the one developed by Neufeld (Austrian, 1976). The method of Neufeld gained more importance only in the 1930s. Using this serotyping method, several serotypes were identified by Mörch in Denmark and by Eddy in the USA, giving rise to two different nomenclatures. While the American nomenclature numbered the serotypes by the order of their discovery, the Danish system allowed also for the grouping of serotypes sharing serological properties into serogroups. Based on this approach, serogroups are denoted with a number, and a letter is added to distinguish between the individual serotypes occurring within a serogroup. After some debate, the Danish classification became the globally adopted nomenclature (Henrichsen, 1999).

Pneumococcal serotyping by the Neufeld method was improved by the development of sera able to react with more than one type of polysaccharide (pooled sera). Since only a restricted number of serotypes are responsible for most pneumococcal disease, Sørensen developed an ordered method for using sera, the chessboard system, which enables to spare time and reagents (Sørensen, 1993). However, the quellung reaction technique is still time-consuming when working with large collections of isolates, requires experienced staff, and to have the complete set of sera reveals unaffordable for most laboratories. Therefore, the Neufeld test is more often used in reference and research laboratories (Slotved et al., 2004).

To overcome the issues associated with the Neufeld test, several other serotyping methods became available. One of the most frequently used techniques is the Pneumotest-Latex, developed at the Statens Serum Institute by Slotved et al. (2004). This protocol uses the chessboard system described by Sørensen (1993), but in this case the pooled pneumococcus sera are applied to latex particles. The main advantages of this method over the quellung reaction technique are that less-experienced workers are also able to serotype and that the time required for serotyping is reduced. Assays that discriminate capsular polysaccharides in patient's urine are also of great value, not only for serotyping but also to help in the diagnosis of pneumonia. The serotype specific urinary antigen detection assay (UAD) (Huijts et al., 2013) detect all the 13 serotypes targeted by the most recently available

pneumococcal vaccine, while the Bio-Plex assay (Sheppard et al., 2011) detects these serotypes plus serotype 8. However, the Bio-Plex assay does not allow the distinction between serotypes 6A and 6C, 7F and 7A nor the serotypes composing serogroup 18. Moreover, both tests share the important limitation of not being able to discriminate all the remaining serotypes. Polymerase chain reaction (PCR) based serotyping protocols are also worth consideration. An example of this approach is the sequential multiplex PCR assay developed in the Centers for Disease Control and Prevention (Pai et al., 2006). This method proved to be cost-effective and to greatly reduce the time needed to serotype large collections of isolates.

Pulsed-Field Gel Electrophoresis Macrorestriction Profiling

The begging of pneumococcal genotyping was dominated by PFGE (Ramirez et al., 2015). In this method, the total DNA of each isolate is subjected to digestion with an infrequently cutting endonuclease (e.g. SmaI) and separation by pulsed-field electrophoresis, generating DNA band profiles that can be compared. The relatedness between isolates is inferred by the number of differences in the band profiles (Figueiredo et al., 1995; Tenover et al., 1995). Initially, the comparisons had to be directly ascertained by the operator, but later, bioinformatics tools were developed to assist in the analysis. This last improvement enabled to decrease the time and error associated with data inspection and allowed the comparison of large collection of isolates within laboratories. A frequently used protocol is to apply a cutoff value of 80% to a dendrogram constructed by the unweighted pair group method with arithmetic means (UPGMA) and DICE coefficient. To be considered clonally-related by PFGE, two pneumococcal isolates must show > 80% similarity. The isolates that are 100% similar by this method are considered identical (Carriço et al., 2005; Ramirez et al., 2015).

With the use of PFGE some important traits of pneumococci were recognized. For example, using this typing system, investigators found that only a few pneumococcal clones led to the global emergence of antimicrobial resistance in pneumococci and that capsular switching occurs naturally in pneumococci, independently of vaccine use (Ramirez et al., 2015).

PFGE is of great use for local epidemic and outbreak surveillance studies. The main benefits of FPGE are its high discriminatory power, intra-laboratory reproducibility and relatively low cost. The major limitations are the low portability of data and its labor-intensive and time-consuming character (Sabat et al., 2013).

Multilocus Sequence Typing

MLST is based on the sequence of internal fragments of seven housekeeping genes, namely, *aroE*, *xpt*, *gki*, *gdh*, *spi*, *ddl* and *recP*. The sequence of each gene is compared to those deposited in a public database (http://www.mlst.net/) to assign an allele number. Sequences corresponding to those in the database are assigned the same allele number, while those differing even at a single nucleotide will give rise to a new allele number. For every unique set of alleles, a distinct sequence type (ST) is defined (Ramirez et al., 2015). This method is globally recognized and up to August 11, 2017, more than 13000 STs were already included in the public database.

A frequent approach for the analysis of MLST data is the eBURST method. Using eBURST, the relationships of the isolates analyzed are represented in an unrooted tree constructed considering the number of differences in the STs. The isolates are distributed into clonal complexes (CCs), which have a founder genotype. According to the eBURST model, a CC emerges in the population either due to a fitness advantage or to random genetic drift. The expansion of a genotype is accompanied by its gradual diversification by mutation and recombination. To be part of a CC, isolates need to share at least 6 alleles with one other isolate of the CC. Therefore, some CCs may be composed of isolates sharing no alleles with each other and having no recent genetic relatedness (Feil, 2004). More recently, a new algorithm was proposed to extend that of eBURST. This later method is called goeBURST, uses all eBURST rules, but considers as a last tie-break, the number attributed to the STs. The foundation of this choice is that the most frequent genotypes will be reported first and, therefore, are more likely to present lower ST numbers (Francisco et al., 2009). MLST data can be analyzed using the freely available software PHILOViZ (www.phyloviz.net). This user-friendly program enables to integrate additional information, such as capsular types, antimicrobial susceptibility patterns and presence of virulence factors (Francisco et al., 2012).

Unlike PFGE, MSLT generates data that is easily comparable in a global scale. Moreover, MLST data is highly reproducible between laboratories and the technique is less demanding than that of PFGE (Sabat el al., 2013). Following its introduction, MLST was used as a complement of PFGE, but since MLST results reinforced the findings previously obtained with PFGE and have all the aforementioned advantages, it became the most commonly used typing method in pneumococcal epidemiological studies. Additionally, the results obtained with MLST may be useful for selection of isolates to be studied by WGS (Ramirez et al., 2015).

Other Genotyping Methods

Other genotyping methods are available for the distinction of pneumococcal isolates, but their use has been more limited. Examples are multilocus enzyme electrophoresis (MLEE) and multilocus variable number of tandem repeat analysis (MLVA). In MLEE, cell lysates go through electrophoresis and multiple assays with specific enzymes. Protein polymorphisms are distinguished based on the position of the resultant staining in the gel. (Selander et al., 1986). MLVA is based on the comparison of repetitive DNA sequences from multiple loci. Pneumococcal isolates may present different number of repeat sequences and these sequences may differ is size (Koeck et al., 2005). Online databases are available to compare MLVA results between laboratories (e.g. http://www.mlva.net/spneumoniae/default.asp).

Antimicrobial Therapy and Resistance in *S. pneumoniae*

Antimicrobial Therapy

In Portugal, empirical antimicrobial therapy of adult patients with CAP not requiring hospitalization is made with β-lactams, more specifically, with amoxicillin. Patients with comorbidities or patients that have taken antimicrobials in the past 3 months are recommended to receive a combination of amoxicillin with a macrolide (i.e. clarithromycin or azithromycin). The use of wide spectrum antibiotics, such as cephalosporins and fluoroquinolones, is restricted to hospitalized patients with severe pneumonia (Direção-Geral da Saúde, 2011). Antimicrobial therapy of pneumococcal meningitis is usually made with ceftriaxone or cefotaxime.

Vancomycin is added to the treatment for patients who have been in countries where cephalosporin non-susceptibility is significant (Goldblatt and O'Brien, 2015).

Availability of Antibiotics and Emergence of Resistance

Before the availability of antibiotics mortality due to bacteremic pneumococcal pneumonia was > 75%, while after, from the 1950s to 1970s, mortality declined to < 30% (Klugman, 1990). Antimicrobial treatment of pneumococcal infections was initially made using optochin. However, this approach did not last for too long, for a number of reasons: optochin is specific for pneumococci, its use in clinical doses leads to the emergence of resistant strains, and optic toxicity is a frequent and serious secondary effect. Later, sulfapyridine, a sulfanilamide derivate, was used for the treatment of pneumococcal infections, until its replacement by penicillin in the 1940s. Fleming discovered penicillin in 1928, but since it was difficult at the time to acquire enough amounts of penicillin for an effective treatment and because sulfapyridine was an effective drug, the importance of penicillin for the treatment of pneumococcal infections remained poorly noticed for more than a decade. The interest in penicillin was renewed in 1940, associated in part with the detection of pneumococci resistant to sulfapyridine (Watson et al., 1993; Wright et al., 2014).

Few years after penicillin started being used as the antimicrobial of choice for the treatment of pneumococcal infections, other antimicrobials effective against pneumococcus started being discovered. Chloramphenicol was discovered in 1947 and erythromycin in 1949. Aureomycin was discovered in 1948 and soon after, in 1952, tetracycline was synthesized from aureomycin. The use of co-trimoxazole (sulfamethoxazole-trimethoprim) occurred later, at the end of the 1960s, while the first to fourth generations of fluoroquinolones were developed from late 1970s to late 1990s (Wright et al., 2014).

Resistant pneumococci for all the aforementioned antibiotics emerged some years after their discovery and use. Resistant strains were in general sporadic during the early 1970s, but became widespread during the 1980s (Klugman, 1990). In the case of penicillin, resistance was demonstrated in the laboratory soon after its extensive use, in the 1940s, but pneumococci remained clinically susceptible to penicillin during the following two decades. The first report of tolerance to penicillin came from

Boston, in 1965, although it did not draw attention at the time. Later, in 1967, Hansman and Bullen reported a resistant isolate from Australia. This was followed shortly after by new cases, again from Australia and also from Papua New Guinea (McGee et al., 2015). Additional reports arose from the USA in 1974, from South Africa in 1977 and from Spain in 1979 (Liñares et al., 2010; McGee et al., 2015). In 1978, 1/3 of 57 isolates causing severe infections in Papua New Guinea were penicillin-non-susceptible (Gratten et al., 1980). In the late 1980s, the fraction of penicillin non-susceptible isolates reached > 40% in Spain (Fenoll et al., 1991) and 70% in Hungary (Marton et al., 1991).

The first report of erythromycin resistant pneumococci is contemporaneous to that of penicillin non-susceptibility (Kislak, 1967). Similarly to penicillin, macrolide resistant pneumococci became globally dispersed with continued use of this class of antibiotics. In the period 2001-2004, erythromycin resistant rates were reported to range from 15% in Latin America to 80% in the Far East from (Felmingham et al., 2007).

Resistance to tetracycline was detected even before the report of penicillin non-susceptibility (Turner, 1963), while the first reports of chloramphenicol resistance in pneumococci occurred in the 1970s (Dang-Van et al., 1978). The emergence pneumococci resistant to co-trimoxazole occurred soon after the availability of this antibiotic, in 1972 (Howe and Wilson, 1972). This later drug has been globally used due to its low cost and effectiveness. Therefore, resistance to co-trimoxazole escalated greatly in many parts of the globe, reaching 50% and > 60% pneumococci in Africa and Asia, respectively (McGee et al., 2015). Like the other classes of antibiotics, resistance to fluoroquinolones did not take too long to emerge (Goldstein and Acar., 1996).

Mechanisms of Resistance

The mechanisms of action of the different antibiotics are diverse, as well as the adaptations of pneumococci to them. β-lactams bind to pneumococcal proteins that are involved in the synthesis of the pneumococcal cell wall, the penicillin-binding proteins (PBPs), inhibiting pneumococcal growth. There are six types of PBPs in pneumococci: PBPs 1a, 1b, 2a, 2b, 2x and 3 (Hakenbeck et al., 1986). The mechanisms

of resistance to β-lactams vary according to the antibiotic, but usually encompass altered *pbp* genes encoding PBP2x, PBP2b or PBP1a (McGee et al., 2015).

Erythromycin prevents protein synthesis due to its binding to the 23S ribosomal RNA. Resistance to this antibiotic can occur by two different mechanisms: target modification and drug efflux. The mechanism of target modification consists in most cases in the methylation of the 23S ribosomal RNA, weakening the affinity of the antibiotic for this structure. This modification results in high-level resistance to macrolides, lincosamides and streptogramins (MLS_B phenotype) (Roberts et al., 1999). In most cases the methylase is encoded in *ermB* gene and much less frequently in the *erm(TR)* gene. Other modifications of the target 23S RNA result from point mutations (Davies et al., 2005). The mechanism of drug efflux consists on the expression of efflux pumps that confer low to moderate resistance to macrolides (14- and 15-member), but not to lincosamides or streptogramin (M phenotype). The efflux pumps are encoded in *mef* genes, in the great majority of cases, *mefA* and *mefE*. The M phenotype has been the most commonly found in Canada, the USA and some countries from Europe and Asia (McGee et al., 2015). Additionally, some isolates can present both mechanisms of macrolide resistance (Felmingham et al., 2007).

Tetracycline targets the 30S ribosome subunit, preventing binding of the tRNA. Resistance to this drug is mediated by the binding of TetM or TetO proteins to the ribosome and the consequent hampering of the association between the antimicrobial and its target (Widdowson et al., 1996). The *tetM* gene is more common that the *tetO* gene (McGee et al., 2015) and is usually carried in transposons that can also harbor the *ermB* gene (Amezaga et al., 2002).

Chloramphenicol targets the enzyme peptidyl transferase, therefore inhibiting bacterial protein synthesis. Resistance to this antimicrobial is due to the acetylation of the drug by an acyltransferase encoded in the *cat* gene (Charpentier and Tuomanen, 2000).

Co-trimoxazole interferes with the biosynthesis of folic acid (Charpentier and Tuomanen, 2000). Resistance to trimethoprim is the result of modifications in the protein dihydrofolate reductase (Adrian and Klugman, 1997), while resistance to sulfonamides results from changes in the protein dihydropteroate synthase (Padayachee and Klugman, 1999).

Fluoroquinolones target DNA gyrase and topoisomerase, which are needed for DNA supercoiling and relaxation. Resistance to this class is the result of mutations in the genes encoding the DNA gyrase or topoisomerase or of the action of drug efflux pumps (McGee et al., 2015).

Pneumococcal Vaccines

Vaccination against *S. pneumoniae* is the best tool to prevent pneumococcal disease, but this measure should be combined with other preventive strategies, such as exclusive breastfeeding during the first 6 months of age, anti-influenza vaccination and control of risk factors for pneumococcal disease (e.g. tobacco smoke; diabetes mellitus and HIV infection) (WHO, 2012; Goldblatt and O'Brien, 2015).

The history of pneumococcal vaccination is almost as old as the history of pneumococci, with pneumococcal vaccines being already marketed in the beginning of the 20th century. The first was a whole-cell vaccine licensed in the USA in 1909, even before a clinical trial. The first trial of a pneumococcal vaccine was conducted in 1911, by Sir Almroth Wright. It was a whole-cell vaccine of unspecified serotypes given to South African gold miners, which reduced the rate of pneumonia during a short period of time. In the 1940s, Colin MacLeod and Michael Heidelberger headed a clinical trial of a pneumococcal polysaccharide vaccine (PPV) targeting four pneumococcal serotypes. This was followed by the licensing in 1947 of two hexavalent PPVs targeting two different groups of serotypes, one for children and the other for adults. However, vaccine adherence was limited due to the preference of clinicians for antimicrobial treatment. Attention was again given to pneumococcal vaccines when Robert Austrian realized that case-fatality rates of pneumococcal disease were still significant despite the availability of antimicrobial treatment (Grabenstein and Klugman, 2012). In 1977, a 14-valent polysaccharide vaccine was licensed, and in 1983, the formulation was extended to encompass a total of 23 polysaccharides (de Paz, 2015).

PPV23 is still in use today, but since plain polysaccharide-based vaccines are not protective in children < 2 years, a different type of pneumococcal vaccine was developed specifically for this age group. In this new type of pneumococcal vaccine,

the polysaccharides are conjugated to carrier proteins, eliciting a different immune response. The development of pneumococcal conjugate vaccines (PCVs) followed the previous knowledge obtained with the development of a successful conjugate vaccine against *Haemophilus influenzae* type b (Adams et al., 1993).

Even though both PPVs and PCVs rely on the polysaccharides included in their formulations to trigger an immune response that is specific to these antigens (Durando et al., 2013), strictly polysaccharide-based vaccines trigger a T cell-independent immune response, not fully functional in young children, while PCVs trigger a T cell-dependent immune response, already functional in infants (Pollard et al., 2009). PPVs stimulate B-cell responses by cross-linking the B-cell receptors. The B cells differentiate into plasmocytes, but this process does not promote proliferation of serotype-specific B cells nor the formation of serotype-specific memory B-cells. In contrast, when polysaccharides are conjugated with carrier proteins, the polysaccharides bind B cells and the proteins are processed within the B cells. The resulting peptides are presented to helper T cells, which are primed by antigen-presenting cells that also processed the carrier proteins. This T cell-dependent response leads to the formation of antibodies that are highly specific and functional, and an anamnestic response is generated with subsequent doses of the vaccine (Durando et al., 2013). Three different pneumococcal conjugate vaccines were licensed since the beginning of the 21st century, PCV7, PCV10 and PCV13, targeting 7, 10 and 13 serotypes, respectively (Ramirez, 2014). Table I.1 shows the main features of PPV23 and of the three PCVs.

Table I.1 Main features of PPV23, PCV7, PCV10 and PCV13.

Vaccine	Targeted Serotypes	Manufacturer/ Trade Name	Year of Licensure
PPV23	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F	Merck & Co / Pneumovax™ 23 Lederle Laboratories (Pnu-Imune™ 23) Sanofi Pasteur / Pneumo 23™	1983 (USA) 1983 (USA) 1987 (Europe)
PCV7	4, 6B, 9V, 14, 18C, 19F, 23F	Wyeth (now Pfizer) / Prevnar™ or Prevenar™	2000 (USA) 2001 (Europe)
PCV10	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	GlaxoSmithKline / Synflorix™	2009 (Europe)
PCV13	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F	Wyeth (now Pfizer) / Prevnar™ 13 or Prevenar™ 13	2010 (Europe; USA)

Based on data from Grabenstein and Klugman (2012) and de Paz et al. (2015).

23-Valent Pneumococcal Polysaccharide Vaccine

Before the availability of PCVs for childhood vaccination, the serotypes included in PPV23 accounted for 85-90% of adult IPD in the USA and other industrialized countries. Also promising was the fact that this vaccine targeted several serotypes frequently associated with antimicrobial resistance (WHO, 2008). However, the efficacy and effectiveness of PPV23 is not well defined and has been under strong debate in the last decades (Huss et al., 2009; Trück et al., 2012; Moberley et al., 2013). Recognizing the inconsistency in the results of randomized control trials and observational studies, the World Health Organization (WHO) stated in its 2008 position paper that the net result of the available studies suggested protection against IPD among healthy young adults and, in a lower extent, among adults ≥ 65 years (WHO, 2008).

In general, PPV23 is believed to prevent IPD but to be less effective in the prevention of non-invasive infections, such as non-invasive pneumococcal pneumonia (Goldblatt and O'Brien, 2015). However, it remains baffling why countries with high PPV23 uptake (~ 70%) and strong surveillance systems for IPD, such as the USA and the UK, are not showing decreases in IPD due to the serotypes targeted exclusively by this vaccine (Sings, 2017). Another concern of PPV23 vaccination is hyporesponsiveness with subsequent doses of the vaccine (Sings, 2017). Therefore, revaccination with PPV23 is usually not recommended (Centers for Disease Control and Prevention, 2010; Tomczyk et al., 2014).

The controversy around the efficacy and effectiveness of PPV23 has resulted in a great variety of national recommendations in Europe (Fedson et al., 2011). In Portugal, PPV23 is available since 1996 (Horácio et al., 2012), but its uptake among adults ≥ 65 years was estimated to be < 10% in one study (Sousa et al., 2009). This situation may be related with the fact that in our country national recommendations for the use of PPV23 have been restricted to specific risk groups only (Direcção-Geral da Saúde, 2005; Direcção-Geral da Saúde, 2010; Direção-Geral da Saúde, 2015b; Direção-Geral da Saúde, 2015c).

7-Valent Pneumococcal Conjugate Vaccine

PCV7, which is composed of the polysaccharides of seven serotypes (Table I.1)

conjugated to the carrier protein CRM197 (cross reactive material 197, non-toxic mutant of diphtheria toxin), was the first pneumococcal conjugate vaccine to become available in the market. This vaccine, also known as Prevenar™, was licensed in the USA in February 2000 and in Europe in February 2001. PCV7 was available for use until its replacement by the homologue vaccine, PCV13, targeting six additional serotypes (Table I.1). PCV7 was licensed in almost 100 countries and included in several national immunization programs (NIPs) (Durando et al., 2013).

The introduction of PCV7 into the NIP of many Western European countries occurred mainly from 2006 to 2008 (Tin Tin Htar et al., 2015). Portugal was one of the few countries from Western Europe not including PCV7 into its NIP (Durando et al., 2013). In Portugal, the vaccine was available through the private market since June 2001 and the initially low vaccine uptake increased throughout the years. In a birth cohort of 2001, the percentage of children with three doses in the first year of life was 23.7%, while in a 2005 birth cohort, was 51.2% (Aguiar et al., 2008a). By 2008, 75% of children with an indication for PCV7 had received the vaccine (Horácio et al., 2012).

PCV7 schedules varied by country. Common regimens were 3+1, 2+1 and 3+0 (Durando et al., 2013). In Portugal, the recommendation was the 3+1 schedule, to be given at 2, 4, 6 and 18 months of age or 3, 5, 7 and 18 months of age (Aguiar et al., 2008a).

The use of PCV7 led to important changes in the dynamics of pneumococcal infections. Several countries reported decreases in the incidence of total IPD in children (Feikin, et al., 2013). In the USA, where PCV7 serotypes accounted for the great majority of IPD, the incidence of total IPD in children < 5 years decreased by 76%, from 98.7 cases per 100,000 population in the pre-PCV7 period, to 23.6 cases per 100,000 population in the post-PCV7 period. After 7 years of PCV7 use, a 100% reduction of PCV7-type IPD was noted in children in this country (Pilishvili et al., 2010). The incidence of total IPD also declined in children in Europe from the pre-PCV7 period to the post-PCV7 period. Examples are the decreases noted in Norway (77/100,000 to 20/100,000) (Steens et al., 2013), Denmark (55.1/100,000 to 25.9/100,000) (Harboe et al., 2014) and France (30.3/100,000 to 24.6/100,000) (Lepoutre et al., 2015). These decreases were due to declines in the incidence of IPD due to PCV7 serotypes, which became very low or null in children in those countries.

Remarkably, reduction of vaccine-type IPD was not only felt among vaccinated children, but also across the surrounding non-vaccinated population, including adults. This phenomenon, known as "herd protection" occurs with the elimination of vaccine serotypes from the nasopharynx of vaccinated children and the interruption of their transmission to close contacts (Moore and Whitney, 2015). In the USA, the incidence of IPD due to PCV7 serotypes decreased among adults within the first few years of PCV7 use in children. In this country, among adults aged ≥ 65 years, IPD caused by PCV7 serotypes declined from 33 in 100,000 population in 1998-1999 to 24 in 100,000 population in 2001 (Whitney et al., 2003). Herd protection was also noticed among adults from Europe. For example, in Denmark, among adults ≥ 65 years, the incidence of IPD due to PCV7 serotypes declined from 27.1 cases per 100,000 population in the pre-PCV7 period to 14 cases per 100,000 population, in the post-PCV7 period (Harboe et al., 2014). The UK (Miller et al., 2011b), France (Lepoutre et al., 2015) and Norway (Steens et al., 2013) also registered declines in IPD due to PCV7 serotypes in adults. In Portugal, PCV7 serotypes were not highly prevalent in adult IPD in the pre-PCV7 period (30.5% in 1999-2003), but they still declined in proportion after PCV7 introduction (16.6% in 2005) (Aguiar et al., 2008a).

Unfortunately, if on the one hand reductions of PCV7 serotypes in carriage of vaccinated children has resulted in decreases of IPD due to these serotypes across the entire population, on the other, the niche left available was promptly occupied by non-PCV7 serotypes (Frazão et al., 2005), resulting in serotype replacement in carriage and disease (Feikin, et al., 2013). In fact, the substitution of vaccine serotypes by non-vaccine serotypes in carriage had been detected in clinical trials conducted before PCV7 availability, making serotype replacement in disease an existing fear (Obaro et al., 1996). Since not all pneumococcal serotypes have a high invasive disease potential (Sá-Leão et al., 2011), the magnitude of serotype replacement in disease was generally lower than that reported for carriage (Weinberger et al., 2011). The net result of serotype replacement in disease varied by geographical region. Most countries reported an overall decrease of IPD incidence, while a few, such as Spain and the UK, reported an overall increase of IPD in some age groups (Weinberger et al., 2011). In the post-PCV7 period, non-PCV7 serotypes 7F and 19A were frequently reported as emerging serotypes in IPD (Moore and Whitney, 2015; Tin Tin Htar et al., 2015).

The hypothesis of an "ecological niche left open" is the most accepted explanation for the increase of non-PCV7 serotypes in carriage and disease. However, other situations may have helped in the emergence of non-vaccine types (NVTs) in disease in the post-PCV7 period, such as secular trends of particular serotypes, incorrect antimicrobial drug use and capsular switching (Moore and Whitney, 2015). Periodic changes in the incidence of IPD due to particular serotypes have been documented and this may be a confounding factor in surveillance studies assessing vaccine impact in IPD (Fenoll et al., 2009; Harboe et al., 2010). Inappropriate antimicrobial drug use is suspected to have played a role in the emergence of non-PCV7 serotypes in the post-PCV7 period, since serotype 19A is highly associated with antimicrobial resistance. Moreover, this serotype increased not only in countries using PCV7 (Aguiar et al., 2008a; Pilishvili et al., 2010; Miller et al., 2011b), but also in those not adopting the vaccine (Choi et al., 2008), strengthening the idea that PCV7 pressure was not the sole reason behind the increase of non-PCV7 serotypes in the post-PCV7 period. Capsular switching occurs naturally in pneumococci (Coffey et al., 1991; Coffey et al., 1998) and there was a concern that clones expressing PCV7 serotypes would change their capsules with serotypes not targeted by PCV7, resulting in vaccine escape. This indeed happened in some cases (Brueggemann et al., 2007), but it still did not account for all the increase of non-PCV7 serotypes in disease in the post-PCV7 period.

An additional theory was raised to explain the increase of non-PCV7 serotypes in carriage. According to this theory, the decrease in prevalence of PCV7 serotypes among vaccinated children resulted in the "unmasking" of non-PCV7 serotypes, already present in carriage but in lower proportions (Lipsitch, 2001). Since co-colonization exists (Valente et al., 2016), this hypothesis is possible. However, this situation can only be a minor contributor to serotype replacement in carriage for several reasons: first, it would imply higher frequencies of co-colonization than those generally reported (Valente et al., 2016); second, co-colonization would have to be caused in a frequent basis by both PCV7 and non-PCV7 serotypes; and third, it does not explain the increase of non-PCV7 serotypes in disease (Weinberger et al., 2011).

Given that five of the seven serotypes targeted by PCV7 are associated with antimicrobial resistance (Dagan and Klugman, 2008), a decrease in antimicrobial

resistance rates was expected in countries using PCV7. A decrease in penicillin non-susceptibility was frequently reported among isolates responsible for IPD in children (Aguiar et al., 2010a; Pilishvili et al., 2010; Pérez-Trallero et al., 2009), but this was not reproduced in all regions for adults (Horácio et al., 2012; Pérez-Trallero et al., 2009). The increase of serotype 19A was the main barrier to the expected reduction in antimicrobial resistance after PCV7 availability (Dagan and Klugman, 2008).

Following PCV7 use in children, some studies from the USA showed an overall decline in mortality (Tsigrelis et al., 2008; Pulido and Sorvillo, 2010), while another found a reduction in the incidence of death, but an increase in case-fatality rates (Lexau et al., 2005). A study from Spain showed overall unaltered case fatality rates (15% vs 17%) (Burgos et al., 2013), but found that IPD was associated with higher rates of septic shock in the post-PCV7 period (19% vs 31%).

The serotypes targeted by PCV7 were chosen based on their high frequency in pediatric IPD in North America. In the pre-PCV7 period, these serotypes accounted for ~ 80% of IPD in the USA and Canada. However, PCV7 serotypes represented much smaller fractions of IPD in children in other continents. For example, these serotypes accounted for < 60% of IPD in Europe, ~ 50% in Latin America, and only ~ 30% in Asia (Hausdorff et al., 2000). Additionally, serotype replacement lowered the potential coverage of PCV7 in several countries (Tin Tin Htar et al., 2015). Therefore, the introduction of PCV10 and PCV13 (Table I.1), targeting previously frequent or later emerging serotypes, was an important mark in the history of pneumococcal disease prevention.

10 and 13-Valent Pneumococcal Conjugate Vaccines

PCV10, also known as 10-valent pneumococcal non-typeable *Haemophilus influenzae* protein D-conjugate vaccine, PHiD-CV or Synflorix™ uses three different protein carriers: serotype 18C is conjugated to the tetanus toxoid, serotype 19F is conjugated to the diphtheria toxoid and the remaining 8 serotypes are conjugated to a recombinant version of protein D of NTHi (Dagan and Frasch, 2009). PCV13, also known as Prevenar 13™, similarly to PCV7, has all its polysaccharides conjugated to CRM197. The European Commission granted a marketing authorization for PCV10 on March 2009, and for PCV13, on December 2009 (Aguiar et al., 2010a). In the USA,

PCV13 received approval for marketing on February 2010 (Aguiar et al., 2010a), while PCV10 was not licensed (Ramirez, 2014).

In the USA, PCV13 was licensed with the same schedule as PCV7, which enabled a rapid switch from PCV7 to PCV13 and a high uptake of PCV13 following its availability (Moore and Whitney, 2015). In Western Europe, several countries adopted the vaccines into their NIPs almost immediately after the licensing of the new PCVs, most of them to substitute the already included PCV7 (Tin Tin Htar et al., 2015). Most of these countries adopted PCV13 (e.g. Italy, France, Norway and the UK), while a few chosen PCV10 (e.g. Finland and the Netherlands) or both vaccines (e.g. Germany and Sweden) (Tin Tin Htar et al., 2015). Portugal included PCV13 into the NIP of children only in June 2015, with a 2+1 schedule at 2, 4 and 12 months of age (Direção Geral da Saúde, 2015a). Before that, both PCV10 and PCV13 were available for children through the private market. However, PCV13 was the most frequently prescribed PCV since its availability, with estimates of 63% coverage in 2012 (Aguiar et al., 2014). Before the inclusion of PCV13 in the NIP of children in Portugal, this vaccine was also the recommended PCV for children and adolescents belonging to risk groups for IPD. In these cases, PCV13 was administered for free (Direcção Geral da Saúde, 2010).

Similarly to PCV7, the use of the new PCVs has resulted in changes in the incidence of IPD in children and adults in numerous sites. In the USA, the incidence of total IPD declined in all age groups when compared with the incidence expected if PCV7 was still in use in children. In this country, declines in total IPD were of 64% in children < 5 years, 53% in children 5-17 years and 12% to 32% among adults, according to the age group. In the USA, the declines in total IPD were mainly promoted by decreases of serotypes 19A and 7F (Moore et al., 2015). Among countries from Western Europe, initial results were diverse, but in several places adopting PCV13 into their NIPs, a decline in incidence of overall IPD was noted for children and adults. Examples of such places are Norway (Steens et al., 2013), Denmark (Harboe et al., 2014), France (Lepoutre et al., 2015) and the UK (Waight et al., 2015). In Switzerland, where PCV13 was included in the NIP, a decrease in incidence of total IPD occurred for children by 2012, but not for adults. Similarly, in Finland, where PCV10 was the adopted vaccine, a decrease in incidence of total IPD was only noted among children,

while in adults > 64 years the incidence of total IPD remained stable (Tin Tin Htar et al., 2015).

The herd protection noticed in adults suggests an effect of PCV13 in carriage, as was reported in some studies (Gladstone et al., 2015; Steens et al., 2015). However, more time will be needed to evaluate the ultimate impact of PCVs on serotype replacement in carriage and disease. For now, some serotypes have been reported to be emerging as causes of disease. Examples are serotypes 8, 15A, 22F and 24F in countries using PCV13 and serotypes 3 and 19A in countries using PCV10 (Tin Tin Htar et al., 2015). Fortunately, in countries where PCV13 was the adopted vaccine, no 19A-like serotype has been shown to be emerging in the post-PCV13 period (Tin Tin Htar et al., 2015).

Adult Vaccination with PCV13

PPV23 was for several years the only vaccine available for the prevention of adult IPD, but this situation changed in late 2011. In October 2011, PCV13 was licensed in Europe for the prevention of IPD in adults ≥ 50 years and on December 2011, this vaccine was licensed in the USA for the prevention of IPD and pneumonia in adults ≥ 50 years (Durando et al., 2013). In 2013, the European indications were expanded to include all adults ≥ 18 years and in 2015, indications expanded again to include not only the prevention of IPD but also pneumonia. In the USA, PCV13 indication was extended for adults ≥ 18 years in 2016 (Sings, 2017).

Following the indications of PCV13 use in adults, many national recommendations for vaccination of adults with PCV13 started to emerge (Castiglia, 2014). In 2012, the US CDC Advisory Committee on Immunization Practices (ACIP) recommended PCV13 for adults ≥ 19 years having immunocompromising conditions and other risk factors for IPD (Sings, 2017). Later, in 2014, ACIP updated its recommendations to include all adults ≥ 65 yrs, immunocompromised or not (Tomczyk et al., 2014). This later recommendation was based on results of a large randomized placebo-controlled trial that became available in 2014. This study took place in the Netherlands and was called the Community-Acquired Pneumonia immunization Trial in Adults (CAPiTA) (Bonten et al., 2015). The CAPiTA study included 85,000 adults ≥ 65 years and aimed to evaluate the efficacy of PCV13 in the

prevention of vaccine-type pneumococcal CAP and vaccine-type pneumococcal IPD. Results from this study showed that PCV13 was 75% effective in the prevention of PCV13-type IPD and 45% effective against PCV13-type NIPP. ACIP recommendations were also based in the remaining burden of disease caused by PCV13 serotypes in adults after some years of PCV13 use in children. With the continued use of PCV13 in children and adults, the incidence of pneumococcal disease due to these serotypes is expected to decrease in all age groups. Therefore, ACIP recommendations were planned to be revised in 2018 (Tomczyk et al., 2014). In Europe, national recommendations for adult vaccination with PCV13 are highly diverse, with some countries having age-based recommendations and others, risk-based recommendations (Castiglia et al., 2014; Sings, 2017). The preferred vaccine (PCV13 or PPV23) also changes with country (Plosker, 2015). In Portugal, the sequential vaccination with PCV13 and PPV23 is recommended for specific risk groups only (Direção-Geral da Saúde, 2015b).

Since childhood immunization with PCVs confer herd protection to the non-vaccinated population, the need for adult vaccination with PCV13 has been questioned (Musher and Rodriguez-Barradas, 2015). Several studies showed that PCV13 is cost effective in adults (Dirmesropian et al., 2015). However, a recent cost-effectiveness study from England, performed after the availability of the results from the CAPiTA study, concluded that vaccinating immunocompetent adults aged ≥ 65 yrs with PCV13 was efficacious, but not cost-effective (van Hoek and Miller, 2016).

Worldwide Availability of Pneumococcal Conjugate Vaccines

The WHO recommends PCVs to be part of routine childhood immunization programs, especially in countries with high infant mortality rates (WHO, 2012). Until 2010, PCVs were mainly available in high income countries, but this scenario changed in that year, with the support of Gavi, the Vaccine Alliance, and the agreement of PCVs manufactures to supply their vaccines at reduced prices in low income countries (Rodgers and Klugman, 2016). As of November 2016, a total of 139 countries (72%) have included PCV10 or PCV13 into their routine immunization programs. However, middle income countries, which have less support, are struggling to include or to maintain PCVs into their NIPs (IVAC, 2017).

Studies evaluating the impact of the PCVs in developing regions have shown high effectiveness both in the targeted population and in the remaining non-targeted population (herd protection). For example, South Africa adopted PCV13 with a 2+1 schedule and showed reductions in IPD in the post-vaccine period in children and adults, independently of HIV status. An important additional benefit was a reduction in antimicrobial resistance (von Gottberg et al., 2014). Kenya adopted PCV10 with a 3+0 schedule and showed a decline of 30% in the incidence of total IPD in children < 5 years (Rodgers and Klugman, 2016). Besides the positive effect against IPD, other studies showed that overall pneumonia hospitalizations also decreased with the use of PCV10 (Afonso et al., 2013) or PCV13 (Becker-Dreps et al., 2014).

Future of Pneumococcal Vaccines

The availability of PCVs has resulted in major health benefits throughout the World. However, these vaccines may not be the ultimate solution for the prevention of pneumococcal disease. First, there is always a possibility of serotype replacement in disease. In fact, the emergence of serotypes not targeted by the vaccines may even occur without PCVs pressure, meaning that static formulations would not succeed in these situations. The overall impact of PCVs has so far been beneficial, but several NVTs have been shown to have a high invasive disease potential (Sá-Leão et al., 2011) being, therefore, candidates to increase as causes of disease in the future (Ramirez et al., 2015). Second, PCVs are complex to produce and relatively expensive. Therefore, low and middle-income countries will struggle to include PCV10 or PCV13 into their NIPs without financial support (Rodgers and Klugman, 2016). Third, non-PCV13 serotypes may have a significant role in the future, since risk groups for IPD are increasing. Concerning this last point, while the younger fraction of the population is growing in developing countries, the older fraction of the population is increasing in developed countries. Regarding older adults, the WHO estimated that individuals ≥ 60 years will reach 2 billion by 2050 (http://www.who.int/ageing/en/). In the USA, hospitalizations due to pneumococcal pneumonia are expected to increase 96% between 2004 and 2040, and without any intervention, the total cost of pneumococcal pneumonia in the USA is estimated to increase $2.5 billion annually (Wroe et al., 2012).

To overcome the limitations of the currently licensed pneumococcal vaccines, several pharmaceutical companies are working on the development of a new type of pneumococcal vaccine. One approach under test is the development of a vaccine targeting surface epitopes that are expressed by all pneumococci. Examples of pneumococcal proteins that are being considered to be part of these new vaccines are pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC) and pneumolysin (Ply). Another strategy is to combine a common pneumococcal protein with a PCV. Additionally, the development of inactivated whole cell preparations is a possibility and this path is likely to be a globally affordable strategy (Alderson, 2016). Another hypothesis is to increase the valency of the existing PCVs. A 15-valent PCV is under evaluation and includes all serotypes of PCV13 plus serotypes 22F and 33F (Skinner et al., 2011). The development of PCVs targeting the most common serotypes occurring specifically in low income countries is another strategy being pursued. One aim of this later approach is to reduce the overall price associated with the production and distribution of PCVs in these regions (Alderson, 2016).

Until a new pneumococcal vaccine is available, or the current vaccine indications are changed, PCVs will remain in use in most countries. The pressure imposed by pneumococcal vaccination and the use of antibiotics will continue to impact on the dynamics of pneumococci and therefore, it is important to maintain epidemiological surveillance of pneumococcal infections. Such surveillance activities can help to understand if vaccine policies adopted by each country remain up to date and may give important insights concerning the next path to follow in the prevention of pneumococcal disease.

AIMS OF THE THESIS

The availability of pneumococcal conjugate vaccines in the 2000s decade prompted an increased need for epidemiological studies in the countries adopting these vaccines. PCV7 became available in Portugal in June 2001, followed by PCV10 in mid-2009 and PCV13 in early-2010. Contrasting with other countries that implemented universal vaccination of children shortly after or few years after the availability of PCVs, in Portugal, only in 2015 was a PCV (PCV13) included in the national immunization program of children. Until then, PCVs were given through the private market, with a modest but increasing uptake. The main aim of this thesis work was to study the characteristics of pneumococci causing disease in adults in Portugal during the period of PCVs use in children outside the national immunization program. The pneumococcal isolates analyzed in this thesis were recovered from adults with invasive pneumococcal disease or non-invasive pneumococcal pneumonia.

Two studies are presented in chapter II of this thesis (Horácio et al., 2013; Horácio et al., 2016b). In these studies, we determined the serotype and antimicrobial susceptibility of pneumococcal isolates causing invasive disease in adults in Portugal in two different periods - 2009-2011 and 2012-2014 – to continue the epidemiological surveillance study initiated in the laboratory in 1999. We aimed to document new changes in the serotype distribution of isolates responsible for adult IPD and to see how these changes related with the private use PCVs in children. PCV13 became available for adults in the beginning of 2012 and therefore, we also aimed to evaluate the potential coverage of this vaccine in adult IPD. The management of pneumococcal infections depends on antibiotic therapy and since antimicrobial resistance has an association with the serotype, another goal was to evaluate if there were significant changes in antimicrobial susceptibility of pneumococci causing adult IPD in this period.

In chapter III, only one study is presented (Horácio et al., 2016a). In this study, isolates causing adult IPD in Portugal between 2008 and 2011 were analyzed regarding their MLST-defined clonal composition and prevalence of pilus islands 1 and 2. The main aim of this study was to analyze the clonal structure of pneumococci responsible for adult IPD in Portugal in the late post-PCV7 period and early post-PCV10/PCV13

period to better understand the changes occurring in adult IPD during the time of private PCVs use in children. For that purpose, we searched specifically for changes in the prevalence of genetic lineages within each serotype and for evidence of capsular switching. Pilus-like structures are virulence factors of pneumococci that contribute to pneumococcal adhesion to host cells (Moschioni et al., 2010; Bagnoli et al., 2008). It is noteworthy that most of the serotypes targeted by the PCVs available to date present these structures, possibly indicating a role of pili in the success of these serotypes. Decreases in the proportion of the serotypes targeted by PCVs likely resulted in decreases in the prevalence of pili, but one study reported a re-emergence of PI-1 among non-vaccine serotypes after several years of PCV7 use (Regev-Yochay et al., 2010). Therefore, another goal of the study presented in chapter III was to evaluate how the presence and type of pili related with pneumococcal serotypes and genotypes responsible for adult IPD and how the use of PCVs in children may have affected the prevalence of pilus islands 1 and 2 among the isolates causing adult IPD.

Chapter IV is composed of two studies (Horácio et al., 2014; Horácio et al., 2018). In these studies, we evaluated the serotype distribution and antimicrobial susceptibility of isolates causing adult non-invasive pneumococcal pneumonia in Portugal in the periods 1999-2011 and 2012-2015, respectively. The aims were similar to those of chapter II for adult IPD. That is, we aimed to characterize changes in the serotype distribution and antimicrobial susceptibility of isolates responsible for adult NIPP and to see if the changes detected were possibly the result of the use of PCV10/PCV13 in children. The period chosen for analysis also included the years of PCV7 use in children and therefore another goal was to evaluate the possible impact of childhood vaccination with PCV7 in adult NIPP. Results from the two studies of chapter IV were compared with contemporary results of adult IPD to evaluate if adult IPD data could be used to infer adult NIPP data.

CHAPTER II: INVASIVE PNEUMOCOCCAL DISEASE IN ADULTS IN PORTUGAL – SEROTYPES AND ANTIMICROBIAL SUSCEPTIBILITY

RATIONALE

The study of IPD is important, not only due to the current burden of these infections, but also because isolation of pneumococci from a normally sterile site unambiguously indicates pneumococcal disease. Therefore, changes in the serotype distribution of the pneumococci causing IPD can be used to evaluate the potential impact of pneumococcal vaccines in IPD.

The two studies presented in this chapter describe the serotype and antimicrobial susceptibility of isolates causing IPD in adults (≥ 18 years) in Portugal in two different periods – 2009-2011 and 2012-2014. They continue the epidemiological study initiated in the laboratory in 1999. Concerning previous studies, results mainly preceding PCV7 availability (1999-2002) were reported in Serrano et al. (2004), data from the early post-PCV7 period (2003-2005) was presented in Aguiar et al. (2008a) and results from the late post-PCV7 period (2006-2008) were reported in Horácio et al. (2012).

In summary, the study of Serrano et al. (2004) showed that in 1999-2002 PCV7 serotypes were much less represented among invasive isolates collected from older children and adults (6-60 yrs: 33.0% and ≥ 60 yrs: 35.1%) than among isolates collected from younger children (< 2 yrs: 63.2% and 2-6 yrs: 60.0%). The most frequent serotypes in this period among individuals aged ≥ 60 years were serotypes 3, 14, 1, 8 and 4. Almost 30% of the isolates recovered from patients ≥ 6 years in 1999-2002 were resistant to at least one class of antibiotics (Serrano et al., 2004).

The study of Aguiar et al. (2008a) gave the first evidence that childhood vaccination with PCV7 outside the national immunization program and with a modest uptake (e.g. ~ 43% in 2004) was likely changing the serotype distribution of pneumococci responsible for adult IPD in Portugal. PCV7 was introduced in 2001 and after 2003 there was a 28% decline in the conditional relative risk that adult IPD was caused by a PCV7 serotype. However, when comparing periods 1999-2003 and 2004-2005, they found an increase in the conditional relative risk of serotypes 19A and 7F and a rise in the fraction of isolates that were resistant to erythromycin, tetracycline and simultaneously non-susceptible to penicillin and erythromycin.

The study of Horácio et al. (2012) reinforced the major findings of the previous work. In this study, the period 1999-2003 was considered a pre-PCV7 period in adults because the initial uptake of PCV7 was too low to be relevant and because the stronger changes in the serotype distribution of adult IPD isolates occurred from 2004 onwards. In this study, we found that the proportion of PCV7 serotypes in adult IPD declined from 30% in 1999-2003 to 16% in 2008. From 2004 to 2008, serotypes 1, 7F and 19A increased in adult IPD. The most frequent serotypes in 2006-2008 were serotypes 3, 1, 7F, 19A and 14. In addition, the proportion of isolates resistant to erythromycin increased again in adult IPD (from 14.4% in 2004-2005 to 18.3% in 2006-2008, p = 0.029).

The studies presented in the current chapter reports on the serotype and antimicrobial susceptibility of the isolates causing adult IPD in a time of PCV10 and PCV13 private use in Portugal (Horácio et al., 2013; Horácio et al., 2016b). PCV10 became available for children in mid-2009 and PCV13 in the beginning of 2010. The first study presented in this chapter (Horácio et al., 2013) evaluates isolates causing adult IPD mostly in a period of transition from PCV7 to PCV10 and PCV13. PCV13 became the most frequently used PCV in Portugal following its availability for children (Aguiar et al., 2014). Therefore, the second study of this chapter (Horácio et al., 2016b) aimed to evaluate the possible impact of the relatively moderate uptake of PCV13 in children (~ 63% in 2012) in adult IPD.

The following study was performed by Andreia N. Horácio, Jorge Diamantino-Miranda, Sandra I. Aguiar, Mário Ramirez, José Melo-Cristino and the Portuguese Group for the Study of Streptococcal Infections. Andreia N. Horácio participated in the experimental work by serotyping and performing antimicrobial resistant tests to part of the isolates, performed great part of the statistical analysis and wrote the first version of the "Results" section of the manuscript. The 2009 data were previously included in the studies of two master thesis (one from Andreia N. Horácio and the other from Jorge Diamantino-Miranda).

THE MAJORITY OF ADULT PNEUMOCOCCAL INVASIVE INFECTIONS IN PORTUGAL ARE STILL POTENTIALLY VACCINE PREVENTABLE IN SPITE OF SIGNIFICANT DECLINES OF SEROTYPES 1 AND 5[1]

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Abstract

In Portugal, pneumococcal conjugate vaccines have been administered to children outside of the national immunization plan since 2001. We determined the serotype and antimicrobial susceptibility of 1265 isolates responsible for adult invasive pneumococcal infections (IPD) between 2009 and 2011 and compared the results with previously published data from 1999 to 2008. Serotypes 3 (12.6%), 7F (10.0%), 19A (9.1%), 14 (8.4%), 1 (6.9%) and 8 (6.2%) were the most frequent and together accounted for 53.2% of adult IPD. Serotypes 1 and 5 declined significantly while serotype 34, not included in any vaccine, increased. Taken together, the serotypes included in the 13-valent conjugate vaccine (PCV13) peaked among adult IPD isolates in 2008 (70.2%) and declined since then reaching 53.5% in 2011. The decline in the serotypes included in the 23-valent polysaccharide vaccine since 2007 was also significant but much more modest with 79.2% of the isolates causing IPD in 2011 expressing these serotypes. Since the changes in serotypes causing IPD in adults coincided with the 10-valent and PCV13 introduction in children, it is unlikely that vaccination triggered these changes although it may have accelerated them. The proportion of IPD caused by serotypes included in the 7-valent conjugate vaccine remained stable (19.0%). Both penicillin non-susceptibility and erythromycin resistance increased in the study period, with serotypes 14 and 19A accounting for the majority of resistant isolates.

[1] A *facsimile* of this publication can be found at the "Publications" section of this thesis.

Introduction

Streptococcus pneumoniae (pneumococcus) remains a significant cause of morbidity and mortality throughout the world affecting disproportionally the extremes of life. Prevention of these infections in persons ≥ 2 years belonging to risk groups and particularly among adults ≥ 65 years has relied on a vaccine including 23 of the 94 capsular polysaccharides known in pneumococci. Although older age is a recognized risk factor for pneumococcal disease, in Europe different countries have distinct recommendations regarding the use of the 23-valent polysaccharide vaccine (PPV23), ranging from the absence of national guidelines, to recommendations of universal or risk group vaccination starting at 60 or 65 years (Michel et al., 2010). Perhaps because of an ongoing debate on PPV23 efficacy (Grabenstein, 2012; Paradiso, 2012; Truck et al., 2012), in most European countries there is a low overall uptake of PPV23 (Fedson et al., 2011). In Portugal PPV23 uptake is at the lower end of the spectrum with estimates that approximately 10% of adults ≥ 65 years are vaccinated (Horácio et al., 2012).

The remarkable efficacy of the seven-valent conjugate vaccine (PCV7) against the serotypes included in its formulation resulted in a sharp decline in the proportion of invasive pneumococcal infections (IPD) caused by these serotypes not only in vaccinated children (Aguiar et al., 2010a; Bettinger et al., 2010; Pilishvili et al., 2010; Rodenburg et al., 2010; Miller et al., 2011b; Ingels et al., 2012) but also across the entire population (Horácio et al., 2012). This "herd effect" is attributed to the reduced transmission of these serotypes from children to adults. In Europe, although there were epidemiological changes in the serotypes causing IPD in the non-vaccinated population in all countries where the vaccine was administered, the large reduction in the overall number of invasive infections in adults observed in the USA was not replicated in countries such as Spain, England and Wales and the Netherlands (Pérez-Trallero et al., 2009; Rodenburg et al., 2010; Miller et al., 2011b) where significant increases in non-vaccine serotypes (NVT) occurred.

Since the PCV7 serotypes represented a significant fraction of resistant isolates, vaccination was also anticipated to affect resistance. However, the effect of PCV7 on antibiotic resistance in Europe was variable. While a decrease in penicillin non-

susceptibility was noted in all countries among isolates responsible for pediatric IPD in the post-PCV7 period (Aguiar et al., 2008a; Pérez-Trallero et al., 2009; Aguiar et al., 2010a), such decline was not apparent in adults in Portugal and Spain (Aguiar et al., 2008a; Pérez-Trallero et al., 2009; Horácio et al., 2012).

Serotype 19A has consistently been identified as a dominant non-vaccine serotype but other emerging non-vaccine serotypes differ between geographic locations and also between age groups (Aguiar et al., 2008a; Aguiar et al., 2010a; Bettinger et al., 2010; Pilishvili et al., 2010; Horácio et al., 2012). Even within serotype 19A, different genetic lineages emerge in different geographic locations (Aguiar et al., 2010b). These data highlight the importance of the characteristics of the local pneumococcal population and of local selective forces in conditioning the outcomes of vaccination (Rosen et al., 2011).

On the other hand, it is known that serotypes responsible for IPD may have significant temporal variations in the same geographic region as documented in Spain and Denmark (Fenoll et al., 2009; Harboe et al., 2010), even with limited antibiotic selective pressure and in the absence of PCV use. In addition, the divergent prevalence of the various serotypes in different geographic regions also conditions the potential benefits of vaccination. A much lower prevalence of serotype 1 IPD is documented in the USA than elsewhere (Aguiar et al., 2008a; Fenoll et al., 2009; Pilishvili et al., 2010; Horácio et al., 2012; Muhammad et al., 2013; Regev-Yochay et al., 2013). Although serotype 1 is frequently associated with outbreaks and significant yearly variations of the proportion of IPD caused by this serotype are documented, a considerable fraction of IPD was consistently caused by this serotype in the last decades in Europe (Fenoll et al., 2009; Harboe et al., 2010).

Two new pneumococcal conjugate vaccine (PCV) formulations are now commercially available and used in children. A 10-valent formulation (PCV10) including, in addition to the PCV7 serotypes, serotypes 1, 5 and 7F and a 13-valent conjugate vaccine (PCV13), including all PCV10 serotypes plus serotypes 3, 6A and 19A. The introduction of these vaccines into clinical practice has the potential to once again change the characteristics of pediatric IPD, with initial data showing the capacity of PCV13 to blunt or even reverse the rise of some of the most successful

serotypes that have emerged as causes of pediatric IPD since the introduction of PCV7 (Miller et al., 2011a; Kaplan et al., 2013; Picazo et al., 2013).

PCV13 was recently licensed for use in adults ≥ 50 years and this was soon followed by a recommendation in the USA for its use in adults with immunocompromising conditions (Centers for Disease Control and Prevention, 2012). Approval of PCV13 for adults was based on immunogenicity studies and the results of a large study that is currently underway in the Netherlands (Hak et al., 2008) comparing it to placebo for the prevention of vaccine-serotype community acquired pneumonia in adults are expected to become available in late 2013. The observed benefits of conjugate vaccines in children launched a discussion about the potential benefits of vaccinating the adult population with these vaccines instead of PPV23 (Musher et al., 2011; Grabenstein, 2012; Paradiso, 2012; Truck et al., 2012). Independently of the immunological arguments, the potential benefits of adult vaccination with either PCV13 or PPV23 are a moving target since secular trends in pneumococcal serotypes and the herd effect provided by PCV7, and now also hoped for the use of PCV13 in children, would be expected to reduce the importance of the serotypes included in conjugate vaccines in adult IPD.

In Portugal PCVs were not included in the National Immunization Plan but there has been a steady increase in PCV7 uptake since 2001, reaching 75% of children ≤ 2 yrs in 2008 (Aguiar et al., 2008a). The expanded valency PCVs for childhood vaccination – PCV10 and PCV13 – became available in mid-2009 and early-2010, respectively. In previous studies, we showed that significant changes in the serotypes causing IPD in children followed PCV7 availability (Aguiar et al., 2008a; Aguiar et al., 2010a) and that there was evidence for a herd effect in the adult population (Aguiar et al., 2008a; Horácio et al., 2012). This study aimed at documenting the continued changes on serotype distribution and antimicrobial resistance in different adult groups and evaluating the proportion of potentially vaccine preventable adult IPD in Portugal immediately prior to the approval in January 2012 of PCV13 use in adults > 50 yrs.

Materials and Methods

Ethics Statement

Case reporting and isolate collection were considered to be surveillance activities and were exempt from evaluation by the Review Board of the Faculdade de Medicina da Universidade de Lisboa.

Bacterial Isolates

Since 1999, the Portuguese Group for the Study of Streptococcal Infections has monitored pneumococci causing invasive infections in Portugal. This is a laboratory-based surveillance system, in which 30 microbiology laboratories throughout Portugal are asked to identify all isolates responsible for IPD and to send them to a central laboratory for characterization. A case of invasive disease is defined by an isolate of *S. pneumoniae* recovered from a normally sterile body site such as blood or CSF. Although the laboratories were contacted periodically to submit the isolates to the central laboratory, no audit was performed to ensure compliance, which may be variable in this type of study. Isolates recovered up to 2008 were previously characterized (Serrano et al., 2004; Aguiar et al., 2008a; Horácio et al., 2012). Only isolates recovered from adult invasive infections, i.e. recovered from patients ≥ 18 yrs, between 2009 and 2011 were included in the present study. One isolate from each patient in each year was considered. All strains were identified as *S. pneumoniae* by colony morphology and hemolysis on blood agar plates, optochin susceptibility and bile solubility.

Serotyping and Antimicrobial Susceptibility Testing

Serotyping was performed by the standard capsular reaction test using the chessboard system and specific sera (Statens Serum Institut, Copenhagen, Denmark). Serotypes were grouped into conjugate vaccine serotypes, i.e., those included in PCV13 (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 19A, 23F) and that comprise all the serotypes found in the lower valency vaccines, those included in PPV23 (all serotypes included in PCV13 except 6A and serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F), and non-vaccine serotypes (NVT). Etest strips (AB Biodisk, Solna,

Sweden) were used to determine the MICs for penicillin and cefotaxime. In 2008, the CLSI changed the recommended breakpoints used to interpret MIC values. Unless otherwise stated we have used the CLSI-recommended breakpoints prior to 2008 (Clinical and Laboratory Standards Institute, 2007) as epidemiological breakpoints that allow the comparison with previous studies. According to these recommendations, intermediate level penicillin resistance was defined as MIC 0.12–1.0 μg/ml and high level resistance as MIC ≥ 2.0 μg/ml. Isolates that fell into either of these classes were designated penicillin non-susceptible. Susceptibility to cefotaxime was defined as MIC ≤ 1.0 μg/ml for non-meningitis cases and an MIC ≤ 0.5 μg/ml for meningitis cases.

Isolates were further characterized by determining their susceptibility to erythromycin, clindamycin, vancomycin, linezolid, tetracycline, levofloxacin, trimethroprim-sulfamethoxazole and chloramphenicol by the Kirby-Bauer disk diffusion technique, according to the CLSI recommendations and interpretative criteria (Clinical and Laboratory Standards Institute, 2011).

Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin according to a previously published procedure (Melo-Cristino et al., 2003). Simultaneous resistance to erythromycin and clindamycin defines the MLS_B phenotype (resistance to macrolides, lincosamides and streptogramin B) while non-susceptibility only to erythromycin indicates the M phenotype.

The prevalence of the various serotypes was compared with already published data from 1999–2008 (Serrano et al., 2004; Aguiar et al., 2008a; Horácio et al., 2012). We established previously that no significant changes in serotype distribution occurred until 2003 in adult IPD and have therefore considered 1999–2003 as the pre-vaccine period (Aguiar et al., 2008a).

Statistical Analysis

Simpson's index of diversity (SID) and respective 95% confidence intervals (CI95%) was used to measure the population diversity (Carriço et al., 2006). Adjusted Wallace (AW) coefficients were used to compare two sets of partitions (Severiano et al., 2011). The calculation of these indices was done using the online tool

www.comparingpartitions.info. Differences were evaluated by the Fisher exact test and the Cochran-Armitage test was used for trends with the false discovery rate (FDR) correction for multiple testing (Benjamini and Hochberg, 1995). A $p < 0.05$ was considered significant for all tests.

Results

Isolate Collection

Between 2009 and 2011 a total of 1265 isolates were recovered from normally sterile sites: 448 in 2009, 404 in 2010 and 413 in 2011. Isolates were recovered from blood (n = 1121, 88.6%), CSF (n = 97, 7.7%), pleural fluid (n = 30, 2.4%), peritoneal fluid (n = 10, 0.8%) and other normally sterile sites (n = 7, 0.5%). Regarding age distribution, 353 isolates (27.9%) were recovered from patients 18–49 yrs, 272 (21.5%) from patients 50–64 yrs and 640 (50.6%) from patients ≥ 65 yrs.

The 1265 isolates recovered in 2009–2011 are in line with the 1100 isolates recovered in 2006–2008 and reported previously (Horácio et al., 2012). This suggests that the surveillance network is stable and that no major changes are affecting IPD reporting in the two periods. However, although unlikely, we cannot completely exclude the possibility that there was an increase in reporting that may have compensated for a potential decrease in IPD incidence.

Serotype Distribution

We detected 50 different capsular types among the 1265 isolates. The most frequent, that accounted for 53.2% of all adult IPD, were serotypes 3 (n = 160, 12.6%), 7F (n = 126, 10.0%), 19A (n = 115, 9.1%), 14 (n = 106, 8.4%), 1 (n = 87, 6.9%) and 8 (n = 79, 6.2%). During the study period (2009–2011), the only significant changes found in individual serotype prevalence after FDR correction were of serotype 1, that decreased from 10.7% to 4.1% (Cochran-Armitage test of trend $p < 0.001$), serotype 5, that decreased from 2.0% to 0% (Cochran-Armitage test of trend $p = 0.003$) and serotype 34, that did not cause any invasive infections in 2009 and 2010 but was detected in 1.9% of the isolates causing IPD in 2011 (Cochran-Armitage test of trend $p < 0.001$).

Figure IIa.1 shows the evolution of vaccine preventable IPD between 1999 and 2011. For the period 1999–2003, defined previously as the pre-PCV7 period (Aguiar et al., 2008a), the results were averaged over the entire period. After the significant decline of IPD caused by PCV7 serotypes between 2004 and 2005, from 30.8% to 16.5% ($p < 0.001$), a steady and low prevalence was seen until 2011. As previously documented (Horácio et al., 2012), in spite of the decrease of PCV7 serotypes, the increase in serotypes 1, 19A and 7F resulted in an overall increase in PCV13 serotypes in the post-

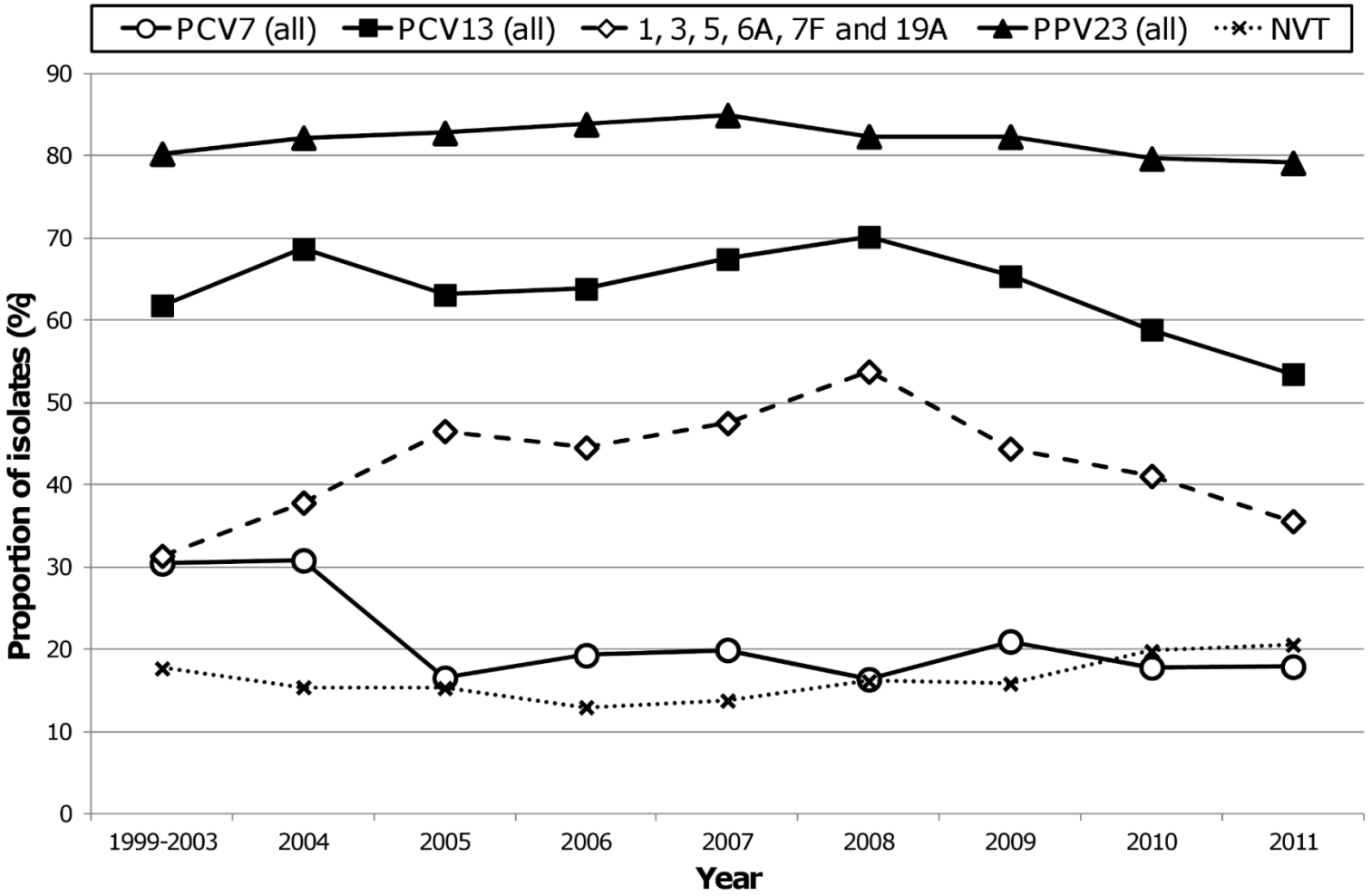

Figure IIa.1 Proportion of isolates expressing serotypes included in pneumococcal vaccines causing invasive infections in adults in Portugal (1999–2011). The data up to 2008 were presented previously (Serrano et al., 2004; Aguiar et al., 2008a; Horácio et al., 2012). The period of 1999–2003, previously identified as the pre-PCV7 period (Aguiar et al., 2008a) was analyzed together.

PCV7 period, from 61.9% in 1999–2003 to 70.2% in 2008 (Cochran-Armitage test of trend p = 0.014). However, 2008 was an inflection point (Fig. IIa.1) and the proportion of isolates presenting PCV13 serotypes started to decline from then onwards such that in 2011 only 53.5% of the isolates presented PCV13 serotypes (from 2008 to 2011, Cochran-Armitage test of trend p < 0.001). This change was mainly driven by a decrease in prevalence of serotypes 1 and 5 from 13.5% and 2.9% in 2008 to 4.1% and 0% in 2011, respectively (Cochran-Armitage test of trend p < 0.001 for both, significant after FDR) (Table SIIa.1).

The proportion of PPV23 serotypes also increased slightly but non-significantly up to 2007. From 2007 onwards there was a slight but significant decrease in the proportion of IPD caused by PPV23 serotypes, from 85.0% in 2007 to 79.2% in 2011 (Cochran-Armitage test of trend p = 0.018). Initially this decline occurred in spite of the increase in PCV13 serotypes that peaked in 2008. Later, the decline of PCV13 serotypes was opposed by an important increase in the proportion of IPD caused by the additional serotypes found in PPV23 but absent from PCV13, from 13.7% in 2008 to 25.9% in 2011 (Cochran-Armitage test of trend p < 0.001). When looking individually at these serotypes, although several increased in frequency, only serotype 8 increased significantly from 3.7% in 2008 to 8.0% in 2011 (Cochran-Armitage test of trend p < 0.002, significant after FDR).

Figure IIa.2 shows the distribution of the individual serotypes included in the conjugate vaccines, stratified by the age group of the patients. Figure IIa.3 shows the distribution of the additional serotypes found in PPV23 that are not included in the conjugate vaccines.

To analyze the serotype diversity within each age group, SIDs were calculated. The serotypes of the isolates causing invasive infections in any of the age groups considered were highly diverse (18–49 yrs [SID: 0.939, CI95%: 0.930–0.947]; 50–64 yrs [SID: 0.949, CI95%: 0.941–0.958]; ≥ 65 yrs [SID: 0.934, CI95%: 0.926–0.943]). The only significant difference was a higher diversity of serotypes in the 50–64 yrs age group relative to ≥ 65 yrs age group (p = 0.013). A similar analysis was performed for determining the serotype diversity in each study year but no significant changes occurred between 2009 and 2011 nor were changes in diversity noted between the 2004–2008 period and 2009–2011 (data not shown).

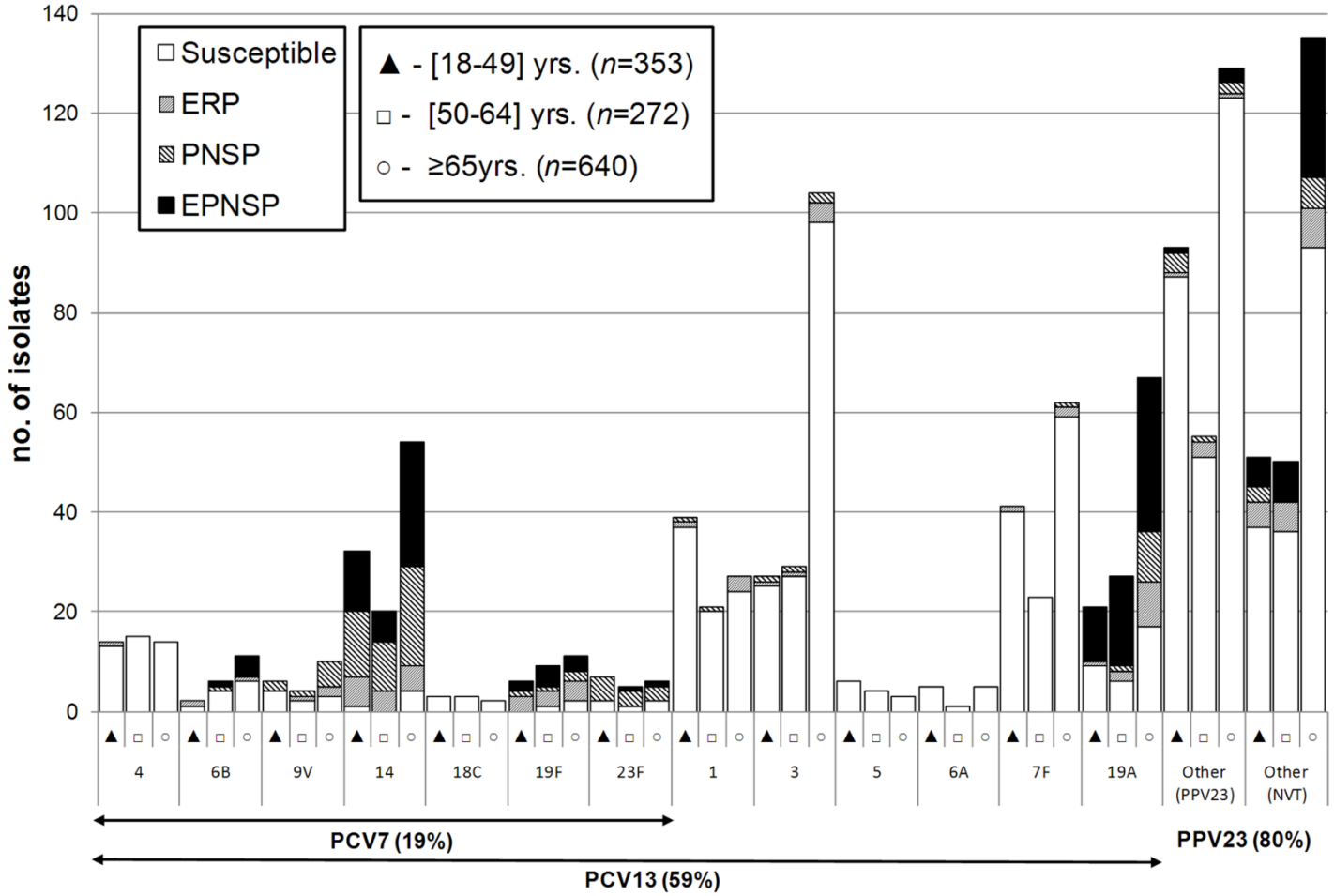

Figure IIa.2 Number of isolates expressing serotypes included in conjugate vaccines causing invasive infections in Portugal (2009–2011). The number of isolates expressing each serotype in each of the age groups considered is indicated. Isolates recovered from patients 18 to 49 yrs are indicated by black triangles. Isolates recovered from patients 50 to 64 yrs are indicated by open squares. Isolates recovered from patients ≥ 65 yrs are indicated by open circles. Isolates presenting both erythromycin resistance and penicillin non-susceptibility (EPNSP) are represented by closed black bars. Penicillin non-susceptible isolates (PNSP) are indicated by dark hatched bars. Erythromycin resistant pneumococci (ERP) are indicated by light hatched bars. Isolates susceptible to both penicillin and erythromycin are represented by white open bars. The serotypes included in each of the conjugate vaccines are indicated by the arrows. NVT – non-vaccine serotypes, i.e., serotypes not included in any of the currently available vaccines (PCV13 and PPV23). Twenty-eight NVT were detected representing 236 isolates as follows: 6C (n=36); 23A (n=20); 12B (n=19); 16F (n=18); 23B and 33A (n=16 each); 15A and 29 (n=15 each); 24F (n=13); non-typable (n=10); 31, 34 and 35F (n=8 each); 7C, 25A and 35B (n=5 each); 21 (n=4); 18A (n=3); 13 and 17A (n=2 each); 7A, 11C, 15F, 24A, 25F, 28A, 28F and 37 (n=1 each).

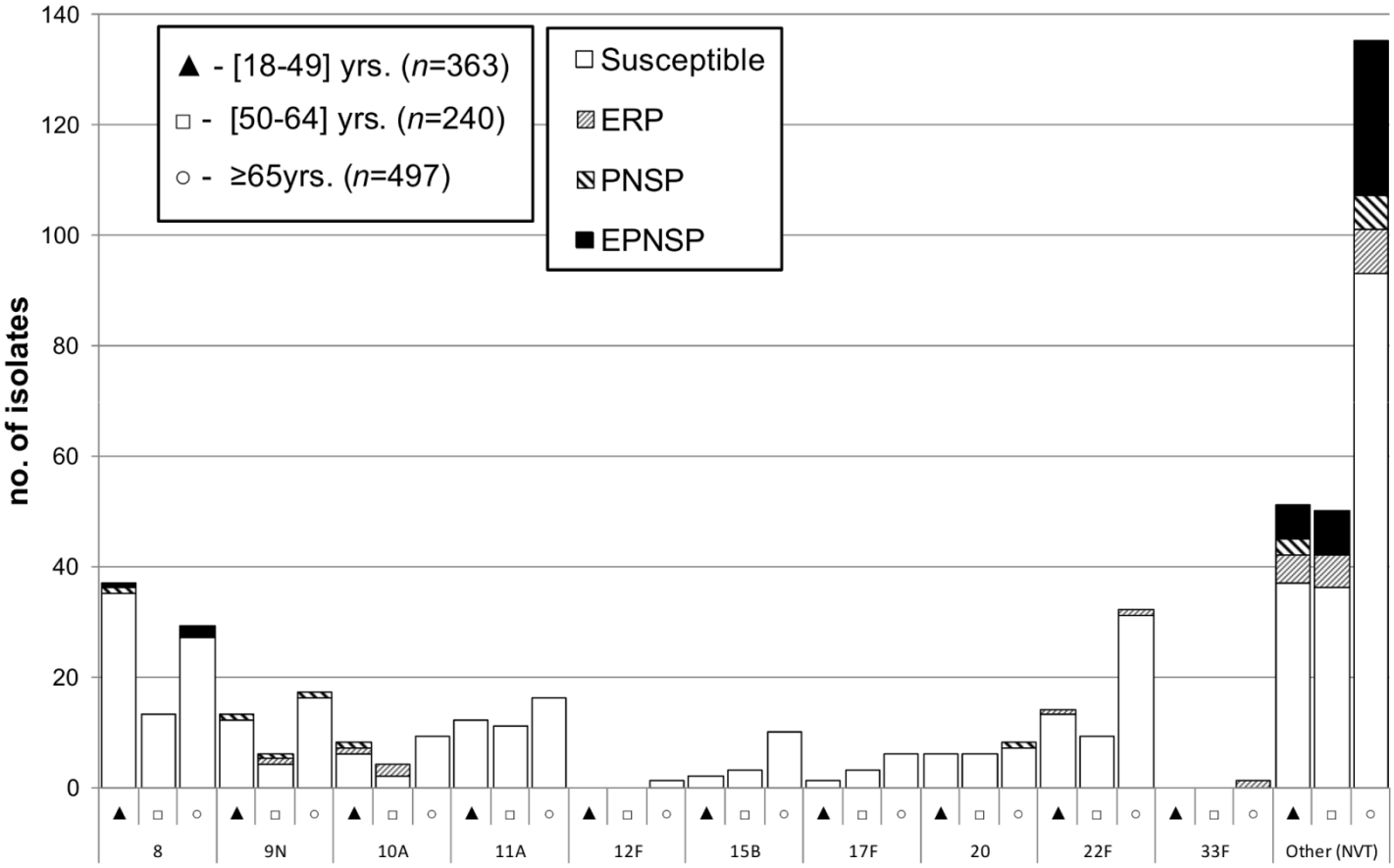

Figure IIa.3 Number of isolates expressing serotypes present in the 23-valent polysaccharide vaccine but not included in conjugate vaccines causing invasive infections in Portugal (2009–2011). See the legend of Figure IIa.1. Out of the 11 serotypes present in the 23-valent polysaccharide vaccine PPV23 but absent from the 13-valent conjugate vaccine PCV13, serotype 2 was not found in our collection.

Although the frequency of each serotype varies according to the age groups considered, only for serotypes 1, 3, 8 and 19A were these differences significant. While the frequency of serotype 1 decreases with age (18–49 yrs – 11.0%, 50–64 yrs – 7.7%, ≥ 65 yrs – 4.2%; Cochran-Armitage test of trend $p < 0.001$), the frequency of serotype 3 increases with age (18–49 yrs – 7.6%, 50–64 yrs – 10.7%, ≥ 65 yrs – 16.3%; Cochran-Armitage test of trend $p < 0.001$). For serotypes 8 and 19A a trend with age was not evident. However, serotype 8 was more frequent in the youngest group (18–49 yrs – 10.5%) than in either of the oldest age groups (50–64 yrs – 4.8%, $p = 0.011$ and ≥ 65 – 4.5%, $p < 0.001$), and serotype 19A showed a higher prevalence in older adults (≥ 65 yrs; 10.5%) than in younger adults (18–49 yrs; 5.9%, $p = 0.019$). Taking together the three years of the study (2009–2011), the proportion of IPD caused by the serotypes included in both conjugate vaccines and in PPV23 was roughly the same in all age groups considered (Table IIa.1).

Table IIa.1 Isolates expressing serotypes included in pneumococcal vaccines (2009-2011).

		No. isolates (%)		
		18-49 yrs	50-64 yrs	≥65 yrs
PCV7	2009	24 (18.2)	21 (22.8)	49 (21.9)
	2010	22 (19.6)	20 (24.7)	30 (14.2)
	2011	24 (22.0)	21 (21.2)	29 (14.1)
PCV13	2009	80 (60.6)	63 (68.5)	150 (67.0)
	2010	65 (58.0)	48 (59.3)	125 (59.2)
	2011	64 (58.7)	56 (56.6)	101 (49.3)
PPV23	2009	104 (78.8)	78 (84.8)	187 (83.5)
	2010	97 (86.6)	66 (81.5)	159 (75.4)
	2011	96 (88.1)	77 (77.8)	154 (75.1)

When analyzing individual serotypes with three or more CSF isolates, a positive association with CSF was found for serotypes 6B ($p = 0.002$), 16F ($p = 0.009$) and 19F ($p = 0.002$), all significant after FDR (Table IIa.S2).

Antimicrobial Susceptibility

Resistance to the tested antimicrobials is summarized in Table IIa.2 and Figures IIa.2 and IIa.3. Overall 266 isolates (21.0%) were penicillin non-susceptible pneumococci (PNSP) – 205 (16.2%) expressed low level resistance (MIC = 0.12–1.0) and 61 (4.8%) high level resistance (MIC ≥ 2 μg/ml).

Table IIa.2 Antimicrobial resistance of the isolates responsible for invasive infections in adults (2009-2011).

	No. resistant isolates (%)[a]		
	18-49 yrs (n=353)	50-64 yrs (n=272)	≥65 yrs (n=640)
PEN	62 (17.6)	58 (21.3)	146 (22.8)
MIC_{90}	1	1	1
MIC_{50}	0.023	0.023	0.023
CTX	14 (4.0)	12 (4.4)	24 (3.8)
MIC_{90}	0.75	0.75	0.75
MIC_{50}	0.023	0.023	0.023
LEV	0 (0)	0 (0)	2 (0.3)
ERY	53 (15.0)	58 (21.3)	134 (20.9)
CLI	40 (11.3)	51 (18.8)	108 (16.9)
CHL	7 (2.0)	11 (4.0)	11 (1.7)
SXT	68 (19.3)	62 (22.8)	117 (18.3)
TET	49 (13.9)	51 (18.8)	118 (18.4)

[a]PEN – penicillin; CTX – cefotaxime; LEV – levofloxacin; ERY – erythromycin; CLI – clindamycin; CHL – chloramphenicol; SXT – trimethoprim/sulphamethoxazole; TET – tetracycline. All isolates were susceptible to vancomycin and linezolid.

Considering current CLSI breakpoints for parenteral penicillin where susceptibility is defined as MIC < 0.06 μg/ml for meningitis cases (Clinical and Laboratory Standards Institute, 2011), 17 isolates (17.5%) from CSF would have been considered resistant and 27 isolates (2.3%) from non-meningitis cases would have been considered non-susceptible – 24 (2.1%) intermediately resistant and 3 (0.3%) fully resistant. Erythromycin resistant pneumococci (ERP) accounted for 19.4% of the isolates (n = 245), of which 194 isolates (79.2%) expressed the MLS_B phenotype and 51 (20.8%) the M phenotype. All isolates were susceptible to vancomycin and linezolid. The simultaneous expression of erythromycin resistance and penicillin non-susceptibility (EPNSP) was found in 13.0% of the isolates.

Resistance to penicillin, erythromycin and clindamycin increased with the age group considered (Table IIa.2). While the increase in erythromycin and clindamycin were statistically supported (Cochram-Armitage test of trend, p = 0.035 and p = 0.039, respectively) the increase in penicillin non-susceptibility was not (p = 0.057). For the other tested antimicrobials no significant differences were noted. The proportions of PNSP and ERP in 2009–2011 were higher than those previously reported (Figure SIIa.1). The proportion of PNSP increased from being previously stable (from an

average value of 16.7% in 1999–2008 to 21.0% in 2009–2011, p = 0.002). On the other hand, there was a consistent increase in the proportion of ERP since PCV7 introduction (from 1999–2003 to 2011, Cochran-Armitage test of trend p < 0.001). Although the overall proportion of ERP has been increasing, when analyzing changes within the study period a significant decrease was noted from 2010 to 2011, from 23.8% to 15.7% (p = 0.005). Similarly, the proportion of PNSP also decreased from 23.8% in 2010 to 19.6% in 2011, but this change was not statistically significant (p = 0.174).

The correlation between serotype and antimicrobial resistance was high. The AW for serotype and penicillin non-susceptibility was 0.539 (CI95% 0.476–0.601), higher than the AW for serotype and erythromycin resistance (0.403, CI95% 0.336–0.470). Together, serotypes 19A and 14 contributed greatly to penicillin non-susceptibility (59.0%) and to erythromycin resistance (53.1%) (Fig. IIa.2). Serotypes included in PCV7 represented 47.4%, 36.7% and 35.8% of PNSP, ERP and EPNSP, respectively, while serotypes included in PCV13 constituted 76.7%, 71.4% and 72.1%, respectively. Considering the serotypes included in PPV23, only a modest increase relative to the PCV13 serotypes is noted (80.2%, 75.1% and 74.5%, of PNSP, ERP and EPNSP, respectively) since most of the remaining resistant isolates express NVTs (Fig. IIa.3). Among the EPNSP expressing NVTs, serotypes 6C (45.2%) and 15A (28.6%) were dominant.

Discussion

The effect of childhood vaccination in the distribution of IPD serotypes in adults was always found to be delayed in relation to the effects seen in children in the countries where the vaccine is available (Aguiar et al., 2008a; Miller et al., 2011b; Demczuk et al., 2012). Given this prior experience with PCV7, the introduction of PCV13 in childhood vaccination in early 2010 in Portugal would only be expected to have an effect in the distribution of serotypes causing IPD in adults around 2011. If one considers the serotypes common to PCV10, then the introduction of PCV10 in childhood vaccination in mid-2009 would raise the possibility that an effect on these serotypes could occur earlier. However, the significant increase in adult IPD in the post-PCV7 period of serotypes 1, 7F (common to both PCVs) and 19A (exclusively found in PCV13) peaked in 2008 (Horácio et al., 2012). While serotypes 7F and 19A remained stable from then onwards, the number of serotype 1 isolates started to decline in 2009 (Table IIa.S1). This was accompanied by a significant reduction of serotype 5 and a strong reduction of serotype 6A (not included in PCV10) in the same period, that were responsible for the overall fall in PCV13 serotypes (Fig. IIa.1). The fact that the decline started in 2009, before PCV13 introduction and shortly after PCV10 became available, strongly argues that the changes seen here were not triggered by the use of PCVs, although they may have been accelerated by PCV use.

Serotype 3 remains the most prevalent serotype in adult IPD after PCV7 introduction and continued to be significantly associated with older adults (Horácio et al., 2012). Similarly, serotype 7F remains the second most frequently identified serotype. Although serotypes 3 and 7F were not as frequent, these were also important among IPD in children (Aguiar et al., 2010a) and were commonly found among patients with pleural parapneumonic effusion of all ages in the same period (unpublished data), attesting to their virulence. In spite of their continued dominance, both serotype 3 and 7F isolates remain mostly susceptible to all tested antimicrobials (Fig. IIa.2), as previously described in Portugal and elsewhere (Serrano et al., 2005; Fenoll et al., 2009).

The increase in serotype 19A as a cause of IPD in both children (Aguiar et al., 2010a) and adults (Horácio et al., 2012) plateaued in the study period in adults with

the overall proportion of IPD isolates expressing this serotype remaining stable at around 9%. Serotype 19A as a whole, did not have an enhanced propensity to cause invasive infections (Sá-Leão et al., 2011), but particular lineages within this serotype were found to have different preferences in their association with the human host (Aguiar et al., 2010b). In Portugal it was shown that the lineage that was expanding was associated with antimicrobial resistance (Aguiar et al., 2010b), as was also seen here (Fig. IIa.2). While no association with particular adult age groups was seen previously for serotype 19A IPD, this serotype was now significantly associated with older adults (≥ 65 yrs) rather than younger adults (18–49 yrs). This could be driven in part by a higher antimicrobial consumption in this age group, a trend that may be emerging in developed countries (Haeseker et al., 2012). However, in Norway the post-PCV7 rise of serotype 19A was mostly dominated by a penicillin susceptible clone (Vestrheim et al., 2012), suggesting that selection for antimicrobial resistance alone cannot explain the post-PCV7 rise of this serotype.

Despite 10 years of PCV7 use in children, serotype 14 was still responsible for a significant fraction of IPD in all adult age groups (Fig. IIa.2) in contrast to what happens elsewhere, where more significant reductions of serotype 14 in adult IPD followed PCV7 use in children (Miller et al., 2011b; Demczuk et al., 2012). Serotype 14 isolates were mostly resistant to either erythromycin or penicillin or both (101/106, 95%) (Fig. IIa.2). A high proportion of penicillin non-susceptibility, erythromycin resistance and erythromycin and penicillin non-susceptibility has been a characteristic of serotype 14 isolates since before PCV7 introduction (Serrano et al., 2004; Serrano et al., 2005; Aguiar et al., 2008a), a feature that was accentuated in the post-PCV7 period in both pediatric and adult IPD (Aguiar et al., 2010a; Horácio et al., 2012).

Serotype 1 traditionally accounts for a higher proportion of pediatric IPD in Europe than in North America. In Portugal serotype 1 was the second most important serotype in adult IPD between 2006–2008 (Horácio et al., 2012). In neighboring Spain, outside the Madrid area where PCV7 was available but not included in the national immunization plan similar to Portugal, an increased importance of serotype 1 in IPD both in the group targeted for PCV7 vaccination as well as in older children and adults was also documented (Marimón et al., 2009). The decline in the importance of

serotype 1 as a cause of IPD with age in adults reported previously (Horácio et al., 2012) was also seen in this period. However, serotype 1 dropped from the second most frequent overall cause of IPD in adults to fifth. As discussed above, the trigger of this decline cannot be solely attributed to a possible herd effect of PCV use in children since it started before vaccination of children with the expanded valency PCVs that include this serotype. Since serotype 1 remains mostly susceptible to antimicrobials (Fig. IIa.2), the continued pressure of antimicrobial use could be invoked to explain this reduction. However, this does not seem plausible since serotypes 3, 4 and 7F, all also included in PCV13 and mostly susceptible to antimicrobials similarly to serotype 1 isolates, remain important and stable serotypes in IPD in adults in Portugal in the post-PCV7 period. Serotypes 1 and 7F were found to have an enhanced invasive disease potential (Sá-Leão et al., 2011) and were thus candidates to increase in prevalence in IPD in the post-PCV7 period, as indeed happened (Horácio et al., 2012). The decline in serotype 1 could be due to unexplained temporal trends that have been known to affect this serotype (Fenoll et al., 2009; Harboe et al., 2010). These temporal trends may have subsequently been accelerated by PCV use.

Two other serotypes with enhanced invasive disease potential – serotypes 5 and 8 (Sá-Leão et al., 2011) – showed opposite trends. Serotype 5 was shown to cause outbreaks in open communities (Vanderkooi et al., 2011) and a significant increase in cases had been noted in 2008, although we have no evidence that these cases correspond to an outbreak. The subsequent decline of serotype 5 isolates could then be the natural dynamics of a putative outbreak. The suggestion that PCV use could have contributed to the decline of serotype 5 is supported by the observation that 2011 is the only year since surveillance started in 1999 when no isolates expressing serotype 5 were detected among IPD cases in adults. Serotype 8 on the other hand is a non-PCV serotype that has been increasing since 2008 and that is now the sixth most frequent serotype in IPD in adults (Fig. IIa.3). Serotype 34 is a NVT that increased in the study period, although it is responsible for a modest number of cases. Although this serotype as a whole was associated with carriage, different lineages expressing this serotype showed distinct capacities to cause invasive disease (Sá-Leão et al., 2011). It is therefore possible that its increase in IPD documented here is driven by a limited expansion of particularly virulent lineages. The other non-PCV serotypes that are

among the ten most frequently found in adult IPD (Fig. IIa.3) have all increased in frequency, although this was not statistically supported, and included the PPV23 serotypes 22F, 11A and 9N and the NVT serotype 6C. The latter was also notable for being frequently resistant to both erythromycin and penicillin. Serotypes 6C and 22F were also found among the most frequent in adult IPD in Canada in 2010 (Demczuk et al., 2012). Since these are not covered by currently available PCVs, these serotypes may emerge as important causes of adult IPD in the post-PCV era.

In spite of the large reduction in the number of PCV7 serotypes, the five serotypes included in this vaccine and that were traditionally associated with resistance (6B, 9V, 14, 19F and 23F), still accounted for a significant fraction of isolates resistant to either erythromycin, penicillin or both (45%), and this proportion declined only slightly from what was seen in 2006–2008 (47%) (Horácio et al., 2012). Both penicillin and erythromycin non-susceptibility, that is concentrated in the serotypes included in PCVs, has risen in adult IPD. The emergence of multiresistant serotype 19A isolates in the post-PCV7 period in adult IPD played a major role in preventing the decline of resistance in IPD in Portugal by compensating the decline of resistant isolates expressing PCV7 serotypes. The significant decrease of erythromycin resistance noted in 2011 may signal a change in this trend, but there were multiple serotypes responsible for this decline and no significant concentration in PCV13 serotypes was noted.

In contrast to our expectations, the use of PCV7 and now of PCV13 has not resulted in further declines of the PCV7 serotypes as causes of adult IPD. The continued importance of these serotypes is in contrast to the massive declines that lead to the almost elimination of PCV7 serotypes as causes of adult IPD in the USA (Pilishvili et al., 2010; Rosen et al., 2011). The reasons behind this difference are possibly multifactorial and may include: 1) differences in the transmission dynamics of these serotypes in the USA and Portugal; 2) differences in the clonal composition or in the selective pressures exerted upon the pneumococcal populations; 3) a relatively slow uptake of PCV7 in Portugal when compared to the USA; and 4) a lower coverage of PCV7 vaccination in children in Portugal. Although we cannot formally exclude the first two possibilities, we believe that their impact would be transient since the pneumococcal population would adapt to these new circumstances. On the

other hand, the later two possibilities are particularly interesting and suggest that in order to obtain the full public health benefits of vaccinating children with PCV13, one should aim at the rapid introduction of the vaccine and at vaccination coverage higher than the 75% currently achieved in Portugal. The proportion of PCV7 serotypes has shown little change since 2005, when the proportion of PCV7 serotypes causing IPD in adults declined from pre-PCV7 levels to its current values. It is therefore likely that the stability of PCV7 serotypes in the intervening six years corresponds to a new steady-state related to the lower vaccination coverage of children in Portugal relative to that in the USA or England and Wales (Miller et al., 2011b; Muhammad et al., 2013).

The recent replacement with the 13-valent formulation for the vaccination of children in Portugal, as has happened in many countries, may equally lead to herd effects in the additional serotypes included in this vaccine. The approval for the use of PCV13 in adults raises the possibility that adult vaccination may not only reduce IPD due to vaccine serotypes in vaccinees but also lead to reduced carriage of the serotypes included in the vaccine, as was seen in children, further compounding the herd effect noted from childhood vaccination. However, even without adult vaccination, continued use of conjugate vaccines in children and the herd effects they produce together with sustained high antimicrobial usage, are likely to drive alterations in the serotypes causing adult IPD that will influence the fraction of potentially vaccine preventable disease in this age group.

Our study was not designed to allow the estimate of the incidence of IPD and it therefore does not evaluate potential changes in incidence with time and in particular since PCV7 introduction. However, the design based on the reporting of all laboratory confirmed IPD cases with isolation of the etiological agent within the surveillance network, the large number of isolates studied, the wide coverage of the country by the network and the stable number of isolates reported in each year, guarantees that the data accurately represents IPD in Portugal and can be used to evaluate changes in the relative importance of the different serotypes. PPV23 availability since 1996 had only minor effects on the proportion of adult IPD caused by PPV23 serotypes but due to its limited use in the country (Horácio et al., 2012) maybe none should be expected. The proportion of infections potentially covered by PPV23 in Portugal remained always above 80% since surveillance was started in 1999, except in 2011 when it

dropped slightly below that level (Fig. IIa.1). This occurred despite significant serotype changes in this period. In contrast, the fraction of adult IPD potentially preventable by PCV13 that had increased diminished in recent years reaching 53.5% in 2011 and may be even lower in patients with co-morbidities (Grau et al., 2012). PCV13 use could still potentially prevent more than half of adult IPD in Portugal at the time it was licensed for use in adults > 50 yrs. The dynamics of the serotypes causing IPD in adults justify the continued surveillance of these infections in order to evaluate the potential coverage afforded by each of the two currently available vaccines with an adult indication.

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Author Contributions

Conceived and designed the experiments: MR and JM-C. Performed the experiments: ANH, JD-M and SIA. Analyzed the data: ANH, MR and JM-C. Contributed with reagents, materials and analysis tools: PGSSI. Wrote the paper: ANH, MR and JM-C.

Supporting Information

Table IIa.S1 Number of isolates expressing serotypes included in the 13-valent conjugate vaccine but not included in the 7-valent conjugate vaccine causing invasive infections in Portugal (2008-2011).

Serotype	No. isolates in each year (%)			
	2008	2009	2010	2011
1	55 (13.4)	48 (10.7)	22 (5.4)	17 (4.1)
3	51 (12.5)	53 (11.8)	59 (14.6)	48 (11.6)
5	12 (2.9)	9 (2.0)	4 (1.0)	0 (0)
6A	6 (1.5)	8 (1.8)	2 (0.5)	1 (0.2)
7F	48 (11.7)	48 (10.7)	35 (8.7)	43 (10.4)
19A	48 (11.7)	33 (7.4)	44 (10.9)	38 (9.2)

Table IIa.S2 Capsular types of the isolates recovered from CSF between 2009 and 2011.

	No. isolates		
Serotype	CSF	non-CSF	OR ($CI_{95\%}$)
3	9	151	0.69 (0.30-1.41)
19A	9	106	1.02 (0.44-2.11)
19F	**7**	**19**	**4.69 (1.62-12.05)**
6B	**6**	**13**	**5.84 (1.78-16.95)**
11A	5	34	1.81 (0.54-4.81)
16F	**5**	**13**	**4.82 (1.32-14.80)**
7F	5	121	0.47 (0.15-1.17)
22F	4	51	0.94 (0.24-2.65)
33A	4	12	4.14 (0.95-14.00)
1	3	84	0.41 (0.08-1.29)
14	3	103	0.33 (0.07-1.02)
10A	3	18	2.04 (0.38-7.17)

[a] Odds ratio (OR) and 95% confidence intervals (CI95%) are shown for serotypes that were represented by at least 3 isolates. [b] In bold are the serotypes with significant p-values after FDR correction.

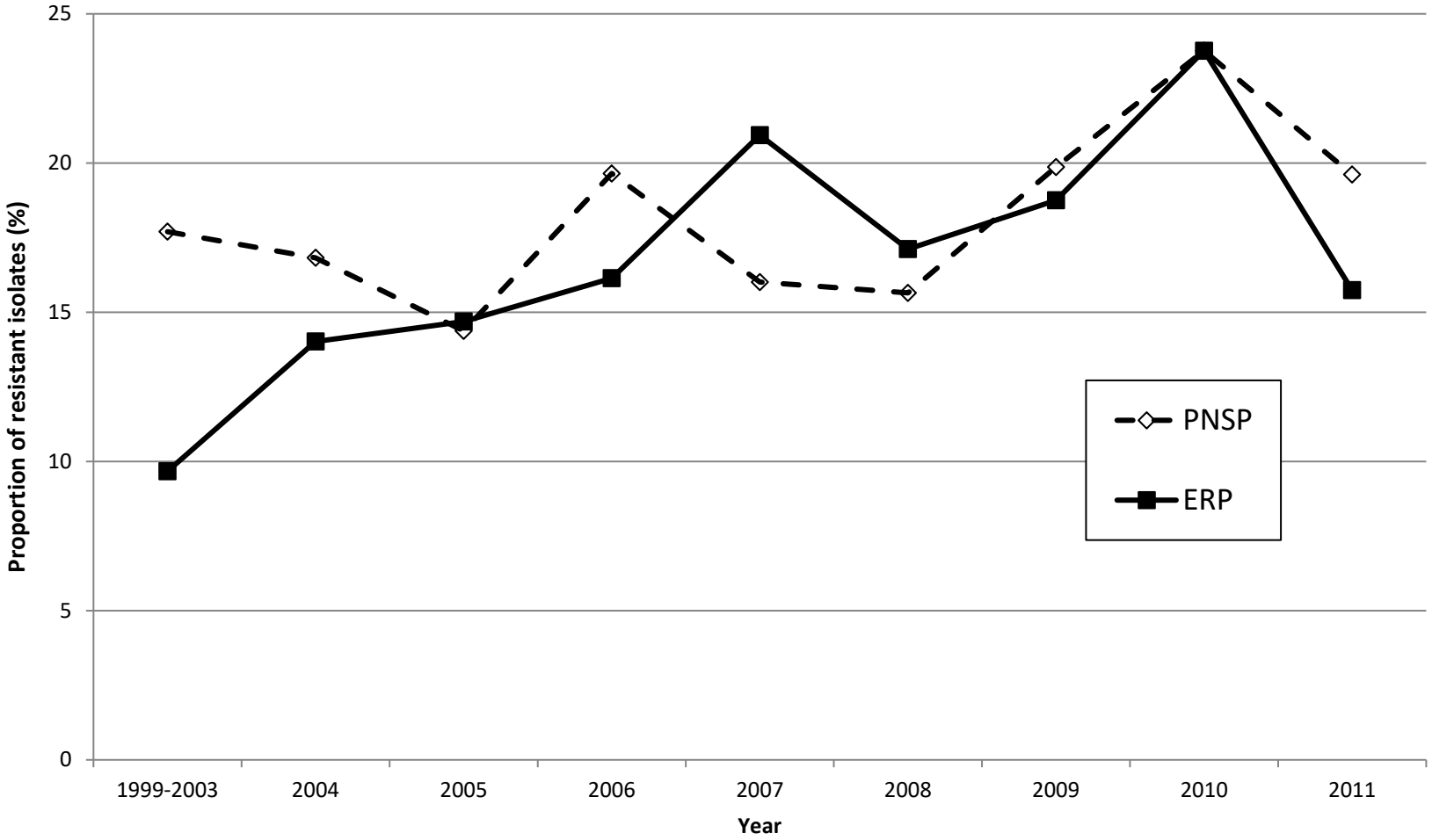

Figure SIIa.1 Proportion of penicillin non-susceptible pneumococci (PNSP) and erythromycin resistant pneumococci (ERP) (1999-2011). The period 1999-2003, previously identified as the pre-PCV7 period, was analyzed together.

The following study was performed by Andreia N. Horácio, Catarina Silva-Costa, Joana P. Lopes, Mário Ramirez, José Melo-Cristino and the Portuguese Group for the Study of Streptococcal Infections. Andreia N. Horácio serotyped a small fraction of the isolates, performed most part of the statistical analysis and wrote the first version of the manuscript.

SEROTYPE 3 REMAINS THE LEADING CAUSE OF INVASIVE PNEUMOCOCCAL DISEASE IN ADULTS IN PORTUGAL (2012–2014) DESPITE CONTINUED REDUCTIONS IN OTHER 13-VALENT CONJUGATE VACCINE SEROTYPES[1]

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Abstract

Since 2010 the 13-valent pneumococcal conjugate vaccine (PCV13) replaced the 7-valent vaccine (PCV7) as the leading pneumococcal vaccine used in children through the private sector. Although, neither of the PCVs were used significantly in adults, changes in adult invasive pneumococcal disease (IPD) were expected due to herd protection. We characterized n = 1163 isolates recovered from IPD in adults in 2012–2014 with the goal of documenting possible changes in serotype prevalence and antimicrobial resistance. Among the 54 different serotypes detected, the most frequent, accounting for half of all IPD, were serotypes: 3 (14%), 8 (11%), 19A (7%), 22F (7%), 14 (6%), and 7F (5%). The proportion of IPD caused by PCV7 serotypes remained stable during the study period (14%), but was smaller than in the previous period (19% in 2009–2011, p = 0.003). The proportion of IPD caused by PCV13 serotypes decreased from 51% in 2012 to 38% in 2014 (p < 0.001), mainly due to decreases in serotypes 7F and 19A. However, PCV13 serotype 3 remained relatively stable and the most frequent cause of adult IPD. Non-PCV13 serotypes continued the increase initiated in the late post-PCV7 period, with serotypes 8 and 22F being the most important emerging serotypes. Serotype 15A increased in 2012–2014 (0.7% to 3.5%, p = 0.011) and was strongly associated with antimicrobial resistance. However, the decreases in resistant isolates among serotypes 14 and 19A led to an overall decrease in penicillin non-susceptibility (from 17 to 13%, p = 0.174) and erythromycin resistance (from 19 to 13%, p = 0.034). Introduction of PCV13 in the NIP for children, as well as its availability for adults may further alter the serotypes causing IPD in adults in Portugal and lead to changes in the proportion of resistant isolates.

[1] A *facsimile* of this publication can be found at the "Publications" section of this thesis.

Introduction

Two types of pneumococcal vaccines are licensed to prevent invasive pneumococcal disease (IPD), both targeting a restricted number of serotypes out of the 94 serotypes currently recognized in *Streptococcus pneumoniae*: strictly polysaccharide based vaccines and polysaccharide-protein conjugate based vaccines (PCVs) (Ramirez, 2014). The first licensed pneumococcal conjugate vaccine was the 7-valent pneumococcal conjugate vaccine (PCV7), which targets serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. PCV7 became available for children in the USA in 2000 and in Europe in 2001. Two additional conjugate vaccines became available more recently: a 10-valent vaccine (PCV10), which includes PCV7 serotypes and serotypes 1, 5, and 7F; and a 13-valent vaccine (PCV13), which includes PCV10 serotypes and serotypes 3, 6A, and 19A. PCVs proved to be highly effective in reducing the number of IPD episodes caused by vaccine serotypes (Pilishvili et al., 2010; Aguiar et al., 2014). Moreover, a decrease in IPD caused by PCV serotypes was also noted in non-vaccinated individuals (a phenomenon termed herd protection) (Horácio et al., 2013; Moore et al., 2015). However, use of PCVs was also accompanied by replacement of vaccine serotypes by non-vaccine types (NVTs) as causes of IPD, both in vaccinated children and in non-vaccinated adults. The overall impact of this phenomenon varied greatly around the world (Pérez-Trallero et al., 2009; Guevara et al., 2014; Harboe et al., 2014; Moore et al., 2015; Waight et al., 2015). The switch to the higher valency vaccines PCV10 and PCV13 also affected emerging serotypes. For instance, serotypes 7F and 19A were reported as emerging in IPD in the post-PCV7 period (Aguiar et al., 2010a; Steens et al., 2013; Guevara et al., 2014; Harboe et al., 2014; Waight et al., 2015) but several studies have already shown that they decrease following PCV13 use (Aguiar et al., 2014; Moore et al., 2015; Waight et al., 2015). A 23-valent strictly polysaccharide vaccine (PPV23) includes 12 of the serotypes found in PCV13 (except6A) and serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F. This vaccine has been used for two decades in older children and adults and has proven efficacy in the prevention of IPD (Moberley et al., 2013).

PCV7, PCV10 and PCV13 became available in Portugal in late-2001, mid-2009 and in early-2010, respectively. However, in contrast to many European countries, in Portugal PCV7 was not included in the national immunization program (NIP) and the

uptake of PCV7 in children increased gradually over time, reaching 75% in 2008 (Aguiar et al., 2008a). PCV13 replaced PCV7 since its availability and has been the most widely used pneumococcal vaccine since then, with estimates of 63% coverage in 2012 (Aguiar et al., 2014). PCV13 received an indication for adults ≥ 50 years in 2012 and in 2013 its indication was extended to all ages, but use of these vaccines in adults in Portugal was believed to be low until 2014. PCV13 was introduced into the NIP for children in 2015, being given free of charge to all children born from January 2015 onwards, with a 2+1 schedule (Direção Geral de Saúde, 2015a). PPV23 is also available in Portugal since 1996, but its uptake among adults is estimated to below (~ 10%) (Horácio et al., 2012). Since 2015, guidelines from the national health authorities recommend vaccinating adults in particular risk groups with both PCV13 and PPV23 (Direção Geral de Saúde, 2015b). However, these groups will constitute a minority of the overall population and there are no guidelines recommending vaccinating adults more broadly with any of the pneumococcal vaccines.

In spite of the gradual increase in PCV uptake in children and the relatively modest coverage, we found significant changes in serotype distribution and antimicrobial susceptibility of pneumococci causing adult IPD that could be attributed at least in part to herd protection. The proportion of adult IPD caused by PCV13 serotypes was highest in 2008 (70%), but a gradual decrease took place until 2011, when only 54% of the isolates causing adult IPD expressed PCV13 serotypes (Horácio et al., 2012 and 2013). In the present study we continued monitoring potential changes in serotype distribution and antimicrobial susceptibility of isolates causing adult IPD after PCV13 received an adult indication and before the introduction of PCV13 in the NIP for children.

Materials and Methods

Ethics Statement

Case reporting and isolate collection were considered to be surveillance activities and were exempt from evaluation by the Review Board of the Faculdade de Medicina of Universidade de Lisboa. The data and isolates were de-identified so that these were irretrievably unlinked to an identifiable person.

Bacterial Isolates

Invasive pneumococcal infections have been monitored in Portugal since 1999 by the Portuguese Group for the Study of Streptococcal Infections (Serrano et al., 2004). This is a laboratory-based surveillance system, in which 31 microbiology laboratories throughout Portugal are asked to identify all isolates responsible for IPD and to send them to a central laboratory for characterization. Although, the laboratories were contacted periodically to submit the isolates to the central laboratory, no audit was performed to ensure compliance, which may be variable in this type of study. A case of IPD was defined by the isolation of pneumococci from a normally sterile fluid, such as blood, pleural fluid or cerebral spinal fluid (CSF). The isolates included in the study were recovered from adult patients (≥ 18 years) with IPD between January 2012 and December 2014. Only one isolate from each patient in each year was included in the study. All isolates were identified as pneumococci by colony morphology, hemolysis on blood agar plates, optochin susceptibility and bile solubility.

Serotyping and Antimicrobial Susceptibility Testing

Serotypes were determined by the standard capsular reaction test using the chessboard system and specific sera (Sørensen, 1993) (Statens Serum Institut, Copenhagen, Denmark). Serotypes were classified into vaccine serotypes, i.e., those included in PCV7 (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F), in PCV10 (all PCV7 serotypes plus serotypes 1, 5, and 7F), in PCV13 (all PCV10 serotypes plus 3, 6A, and 19A) or in PPV23 (all PCV13 serotypes, except serotype 6A and serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F) and non-vaccine serotypes (NVT). Given the high frequency of spontaneous switching between serotypes 15B and 15C we have opted to group

isolates with these serotypes into a single group. Due to difficulties in phenotypically distinguishing isolates of serotype 25A and serogroup 38 these were also grouped together into the 25A/38. Minimal inhibitory concentrations (MICs) for penicillin and cefotaxime were determined using Etest strips (Biomérieux, Marcy l'Étoile, France). In 2008, the CLSI changed the recommended breakpoints used to interpret MIC values. Unless otherwise stated we have used the CLSI-recommended breakpoints prior to 2008 (Clinical and Laboratory Standards Institute, 2007) as epidemiological breakpoints that allow the comparison with previous studies. Isolates were further characterized by determining their susceptibility to erythromycin, clindamycin, vancomycin, linezolid, tetracycline, levofloxacin, trimethroprim-sulfamethoxazole and chloramphenicol by the Kirby-Bauer disk diffusion technique, according to the CLSI recommendations and interpretative criteria (Clinical and Laboratory Standards Institute, 2014). Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin, as previously described (Melo-Cristino et al., 2003). Simultaneous resistance to erythromycin and clindamycin defines the MLS_B phenotype (resistance to macrolides, lincosamides and streptogramin B) while non-susceptibility only to erythromycin indicates the M phenotype.

Statistical Analysis

Simpson's index of diversity (SID) and respective 95% confidence intervals (CI95%) was used to measure the population diversity (Carriço et al., 2006). Adjusted Wallace (AW) coefficients were used to compare two sets of partitions (Severiano et al., 2011). These indices were calculated using the online tool available at http://www.comparingpartitions.info. Differences were evaluated by the Fisher exact test and the Cochran-Armitage test (CA) was used for trends with the false discovery rate (FDR) correction for multiple testing (Benjamini and Hochberg, 1995). A $p < 0.05$ was considered significant for all tests.

Results

Isolate Collection

A total of 1163 isolates were collected from adults with invasive pneumococcal disease between 2012 and 2014: 404 in 2012, 383 in 2013 and 376 in 2014. The majority were recovered from blood (n = 1066, 91.7%) and the remaining from CSF (n = 59, 5.1%), pleural fluid (n = 26, 2.2%), peritoneal fluid (n = 9, 0.8%) and other normally sterile sites (n = 3, 0.3%).

Serotype Distribution

Between 2012 and 2014, a total of 54 different serotypes were identified. The most frequent, which accounted for half of the isolates were serotypes 3 (n = 161, 13.8%), 8 (n = 123, 10.6%), 19A (n = 84, 7.2%), 22F (n = 79, 6.8%), 14 (n = 73, 6.3%), and 7F (n = 61, 5.2%). Figures IIb.1–IIb.3 represent the number of isolates expressing serotypes included in PCVs, the additional serotypes found in PPV23, and the number of isolates expressing NVTs stratified by age group. Serotype diversity was high (2012–2014 SID = 0.944, CI95%: 0.939–0.949). Although, diversity was > 0.93 in all the studied years, there was a small but significant increase in serotype diversity between 2012 (SID = 0.935, CI95%: 0.924–0.945) and 2013 (SID = 0.950, CI95%: 0.942–0.958) (p = 0.019). Serotype distribution varied according to age group but serotype diversity was not different in the three age groups considered (18–49 years, SID = 0.948, CI95%: 0.938–0.958; 50–64 years, SID = 0.945, CI95%: 0.933–0.957; ≥ 65 years, SID = 0.939, CI95%: 0.931–0.946). Only for serotype 1 were the differences in age distribution statistically supported after FDR correction with the proportion of serotype 1 decreasing with age (accounting for 6.5, 2.6, and 0.6% of the isolates recovered from patients aged 18–49 years, 50–64 years and ≥ 65 years, respectively, CA p < 0.001). In contrast, the proportion of IPD caused by the group of additional serotypes found only in PCV13 (3, 6A, and 19A) increases with age (15.2% in 18–49 years, 19.9% in 50–64 years and 24.7% in ≥ 65years, CA p = 0.002, significant after FDR).

When considering serotypes presenting three or more CSF isolates, we found a positive association with CSF for serotypes 19F (p = 0.006) and 23B (p = 0.005), both

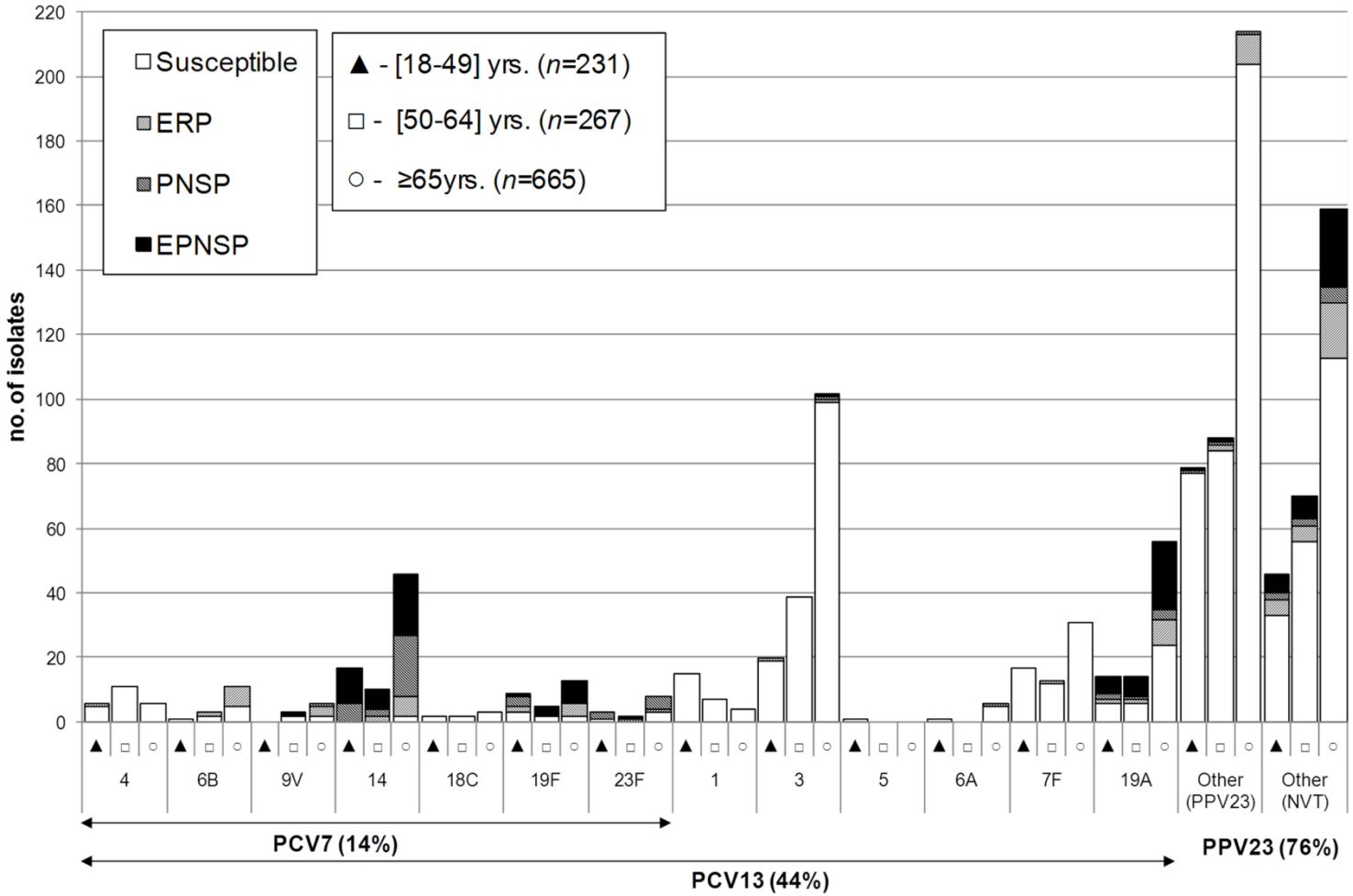

Figure IIb.1 Serotypes of isolates causing invasive pneumococcal disease in adult patients (≥18 years) in Portugal, 2012–2014. The number of isolates expressing each serotype in each of the age groups considered is indicated. Isolates recovered from patients 18–49 years are indicated by black triangles, from patients 50–64 years by open squares, and from patients ≥65 years by open circles. Isolates presenting both erythromycin resistance and penicillin non-susceptibility (EPNSP) are represented by black bars. Penicillin non-susceptible isolates (PNSP) are indicated by dark hatched bars. Erythromycin resistant pneumococci (ERP) are indicated by light hatched bars. Isolates susceptible to both penicillin and erythromycin are represented by white bars. The serotypes included in the seven-valent conjugate vaccine (PCV7) and in the 13-valent conjugate vaccine (PCV13) are indicated by the arrows. NVT, non-vaccine serotypes; PPV23, 23-valent polysaccharide vaccine.

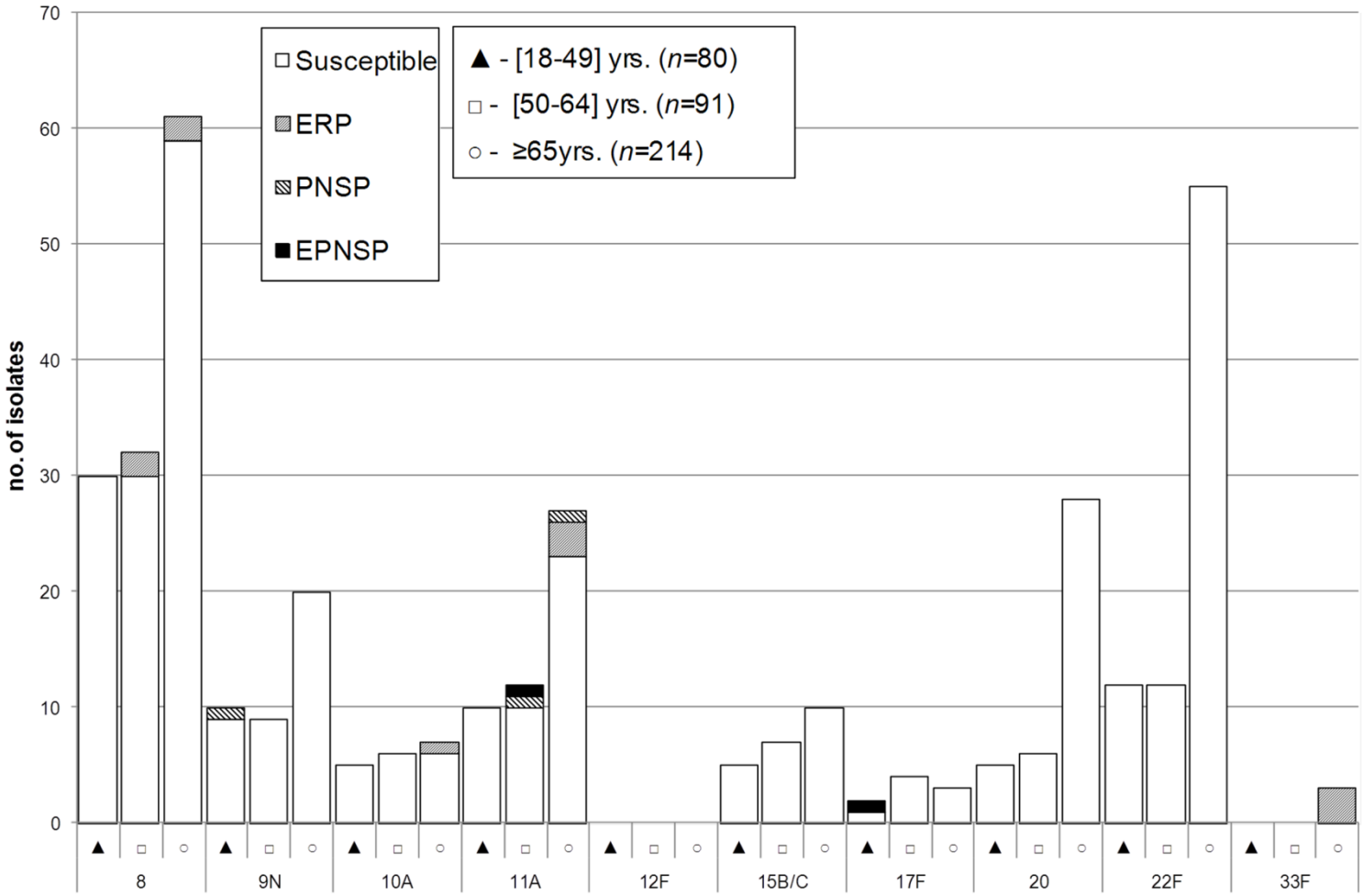

Figure IIb.2 Isolates expressing serotypes present in PPV23 but not included in conjugate vaccines causing invasive pneumococcal disease in adult patients (≥18 years) in Portugal, 2012–2014. See legend of Figure IIb.1 Out of the 11 serotypes present in PPV23 but absent from PCV13, serotype 2 was not found in our collection.

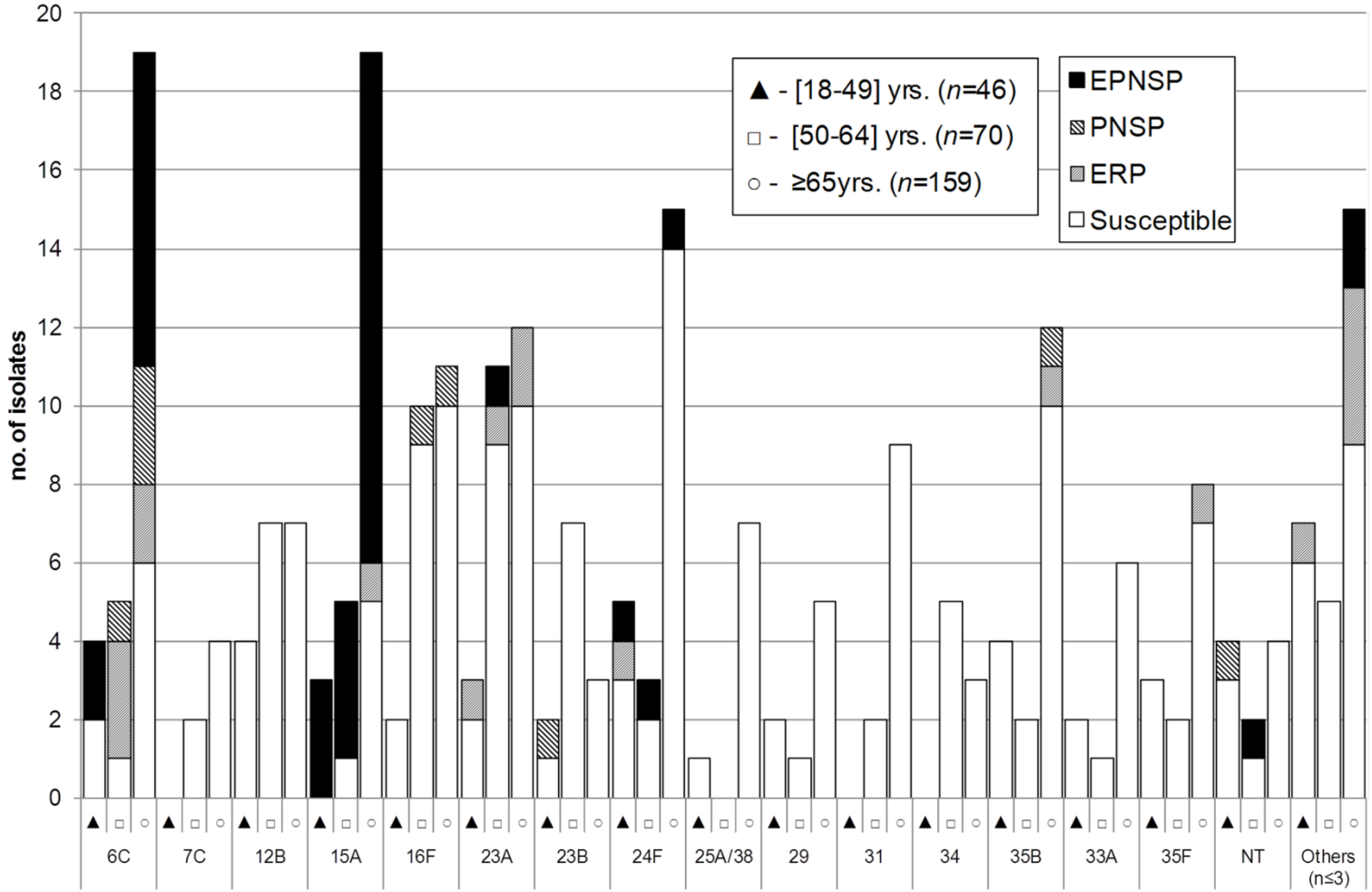

Figure IIb.3 Isolates expressing serotypes not included in any pneumococcal vaccine causing invasive pneumococcal disease in adult patients (≥18 years) in Portugal, 2012–2014. See legend of Figure IIb.1. NT, non-typable. Isolates expressing serotype 25A and 38 could not be distinguished phenotypically and are represented together. Only serotypes including n > 3 isolates are discriminated.

significant after FDR correction (Table IIb.S1). No significant associations with serotype were found for isolates recovered from pleural fluid.

Figure IIb.4 shows the proportion of potentially vaccine preventable IPD during the study period and, for comparison purposes, also from 2008 to 2011 since important changes in serotype distribution initiated in this period (Horácio et al., 2013). Considering the current study period only (2012–2014), the overall proportion of IPD caused by PCV7 serotypes remained stable, while there was a decrease in the proportion of IPD caused by the additional serotypes found in both PCV10 and PCV13 (serotypes 1, 5, 7F; from 11.1 to 4.8%, p = 0.001, significant after FDR) and in PCV13 only (serotypes 3, 6A, and 19A; from 26.5 to 19.9%, p = 0.024, significant after FDR).This resulted in the overall decrease in the proportion of IPD caused by PCV13 serotypes from 51.2% in 2012 to 38.0% in 2014 (p < 0.001, significant after FDR). The proportion of IPD caused by PPV23 serotypes and NVTs did not suffer significant changes during the study period (Figure IIb.4). However, the proportion of IPD caused by the additional serotypes found only in PPV23 (PPV23 add) significantly increased, from 27.2 to 38.0% (p = 0.001, significant after FDR).

When considering the evolution of potentially vaccine preventable IPD in the entire period from 2008 to 2014, there was a decrease in the overall proportion of IPD caused by PCV13 serotypes, although this was temporarily interrupted in 2012, mainly due to a slight increase of serotype 3 (see below).

Table IIb.1 shows the evolution of individual serotypes causing adult IPD from 2008 to 2014. When looking for trends in the proportion of individual serotypes during the current study period (2012–2014), the only significant change that was supported after FDR correction was the decrease in serotype 7F (from 8.2% in 2012 to 4.7% in 2013 and 2.7% in 2014, CA p < 0.001). No significant changes in the proportion of individual serotypes were detected during the study period when stratifying by age group (data not shown).

When considering together data from 2008 to 2014 there were changes (significant after FDR) in the proportion of individual serotypes. There were decreases in the proportion of IPD caused by serotypes: 1 (from 13.4 to 1.9%, CA p < 0.001), 5 (from 2.9 to 0.3%, CA p < 0.001), 9V (from 3.4 to 0.3%, CA p < 0.001) and 19A (from 11.7 to 5.6%, CA p = 0.005). In contrast, there were increases in the proportion of IPD

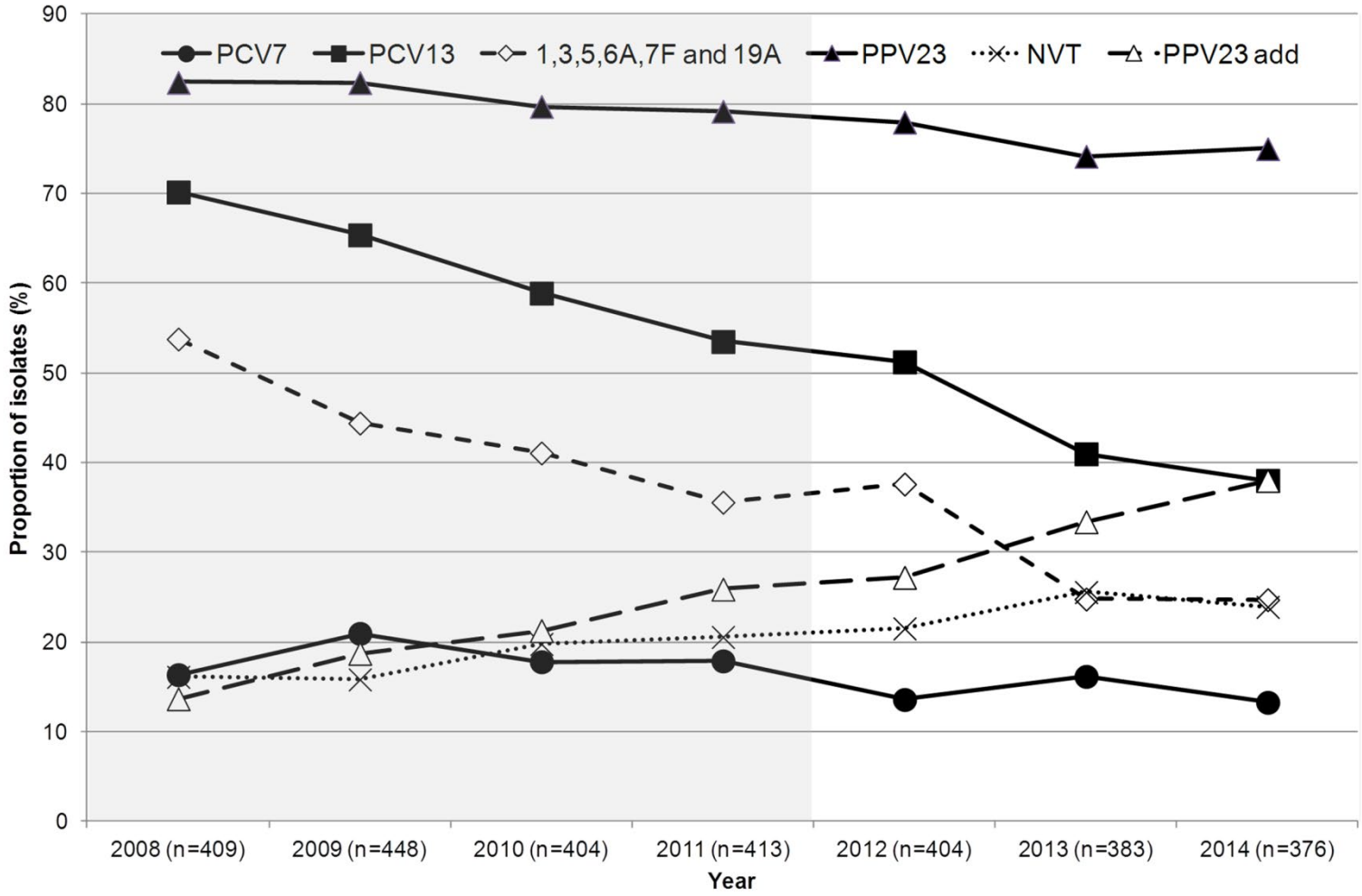

Figure IIb.4 Proportion of isolates expressing serotypes included in pneumococcal vaccines causing invasive pneumococcal disease in adult patients (≥18 years) in Portugal, 2008–2014.
The data up to 2011 were presented previously (Horácio et al., 2012, 2013).

Table IIb.1 Serotypes of the isolates responsible for invasive pneumococcal disease in adult patients (≥18 years), 2008–2014.

Serotype	No. of isolates (%)							CA[a]	CA
	2008	2009	2010	2011	2012	2013	2014	2012-14	2008-14
PCV13									
1	55 (13.4)	48 (10.7)	22 (5.4)	17 (4.1)	12 (3.0)	7 (1.8)	7 (1.9)	0.289	**<0.001**
3	51 (12.5)	53 (11.8)	59 (14.6)	48 (11.6)	66 (16.3)	45 (11.7)	50 (13.3)	0.209	0.613
4	10 (2.4)	12 (2.7)	17 (4.2)	14 (3.4)	6 (1.5)	8 (2.1)	9 (2.4)	0.360	0.352
5	12 (2.9)	9 (2.0)	4 (1.0)	0 (0)	0 (0)	0 (0)	1 (0.3)	0.211	**<0.001**
6A	6 (1.5)	8 (1.8)	2 (0.5)	1 (0.2)	2 (0.5)	1 (0.3)	4 (1.1)	0.315	0.062
6B	1 (0.2)	7 (1.6)	3 (0.7)	9 (2.2)	5 (1.2)	5 (1.3)	5 (1.3)	0.909	0.272
7F	48 (11.7)	48 (10.7)	35 (8.7)	43 (10.4)	33 (8.2)	18 (4.7)	10 (2.7)	<0.001	**<0.001**
9V	14 (3.4)	7 (1.6)	8 (2.0)	5 (1.2)	4 (1.0)	4 (1.0)	1 (0.3)	0.255	**<0.001**
14	29 (7.1)	45 (10.0)	30 (7.4)	31 (7.5)	29 (7.2)	26 (6.8)	18 (4.8)	0.172	0.045
18C	0 (0)	6 (1.3)	1 (0.2)	1 (0.2)	1 (0.2)	4 (1.0)	2 (0.5)	0.588	0.676
19A	48 (11.7)	33 (7.4)	44 (10.9)	38 (9.2)	39 (9.7)	24 (6.3)	21 (5.6)	0.027	0.005
19F	7 (1.7)	13 (2.9)	8 (2.0)	5 (1.2)	9 (2.2)	12 (3.1)	6 (1.6)	0.576	0.956
23F	6 (1.5)	4 (0.9)	5 (1.2)	9 (2.2)	1 (0.2)	3 (0.8)	9 (2.4)	0.005	0.618
PPV23									
8	15 (3.7)	19 (4.2)	27 (6.7)	33 (8.0)	34 (8.4)	43 (11.2)	46 (12.2)	0.081	**<0.001**
9N	10 (2.4)	12 (2.7)	13 (3.2)	11 (2.7)	8 (2.0)	13 (3.4)	18 (4.8)	0.030	0.122
10A	3 (0.7)	8 (1.8)	7 (1.7)	6 (1.5)	2 (0.5)	8 (2.1)	8 (2.1)	0.062	0.294
11A	7 (1.7)	13 (2.9)	10 (2.5)	16 (3.9)	16 (4.0)	18 (4.7)	15 (4.0)	0.974	0.012
12F	0 (0)	1 (0.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	-	0.334
15B/C	8 (2.0)	4 (0.9)	3 (0.7)	8 (1.9)	5 (1.2)	9 (2.3)	8 (2.1)	0.353	0.096
17F	3 (0.7)	2 (0.4)	4 (1.0)	4 (1.0)	5 (1.2)	2 (0.5)	2 (0.5)	0.255	0.981
20	4 (1.0)	8 (1.8)	5 (1.2)	7 (1.7)	14 (3.5)	11 (2.9)	14 (3.7)	0.851	**0.001**
22F	10 (2.4)	17 (3.8)	16 (4.0)	22 (5.3)	25 (6.2)	23 (6.0)	31 (8.2)	0.261	**<0.001**
33F	0 (0)	0 (0)	1 (0.2)	0 (0)	1 (0.2)	1 (0.3)	1 (0.3)	0.959	0.180
NVT[b]									
6C	4 (1.0)	13 (2.9)	13 (3.2)	10 (2.4)	8 (2.0)	14 (3.7)	6 (1.6)	0.757	0.600
15A	4 (1.0)	5 (1.1)	5 (1.2)	5 (1.2)	3 (0.7)	11 (2.9)	13 (3.5)	0.011	**0.002**
23A	6 (1.5)	8 (1.8)	8 (2.0)	4 (1.0)	9 (2.2)	8 (2.1)	9 (2.4)	0.879	0.317
16F	3 (0.7)	8 (1.8)	3 (0.7)	7 (1.7)	13 (3.2)	3 (0.8)	7 (1.9)	0.161	0.234
24F	3 (0.7)	6 (1.3)	5 (1.2)	2 (0.5)	5 (1.2)	9 (2.3)	9 (2.4)	0.241	0.027
12B	10 (2.4)	5 (1.1)	3 (0.7)	11 (2.7)	6 (1.5)	8 (2.1)	4 (1.1)	0.649	0.700
35B	2 (0.5)	5 (1.1)	10 (2.5)	5 (1.2)	6 (1.5)	4 (1.0)	8 (2.1)	0.480	0.222
35F	2 (0.5)	3 (0.7)	2 (0.5)	3 (0.7)	7 (1.7)	4 (1.0)	2 (0.5)	0.110	0.350
23B	4 (1.0)	3 (0.7)	7 (1.7)	6 (1.5)	4 (1.0)	5 (1.3)	3 (0.8)	0.801	0.958
31	2 (0.5)	2 (0.4)	1 (0.2)	5 (1.2)	5 (1.2)	2 (0.5)	4 (1.6)	0.786	0.197
NT	0 (0)	3 (0.7)	4 (1.0)	3 (0.7)	1 (0.2)	3 (0.8)	6 (1.6)	0.042	0.060
33A	1 (0.2)	4 (0.9)	8 (2.0)	4 (1.0)	2 (0.5)	5 (1.3)	2 (0.5)	0.930	0.913
25A/38	2 (0.5)	3 (0.7)	0 (0)	2 (0.5)	3 (0.7)	3 (0.8)	2 (0.5)	0.726	0.583
29	0 (0)	0 (0)	0 (0)	0 (0)	4 (1.0)	2 (0.5)	2 (0.5)	0.433	0.009
34	2 (0.5)	0 (0)	0 (0)	8 (1.9)	3 (0.7)	1 (0.3)	4 (1.0)	0.607	0.148
7C	2 (0.5)	2 (0.4)	2 (0.5)	1 (0.2)	1 (0.2)	4 (1.0)	1 (0.3)	0.944	0.907
18A	6 (1.5)	0 (0)	1 (0.2)	2 (0.5)	0 (0)	3 (0.8)	0 (0)	0.959	0.080
21	3 (0.7)	0 (0)	0 (0)	4 (1.0)	0 (0)	0 (0)	0 (0)	-	0.108
Others[c]	8 (2.0)	1 (0.2)	8 (2.0)	3 (0.7)	7 (1.7)	9 (2.3)	8 (2.1)	-	-
Total	409	448	404	413	404	383	376	-	-

[a]CA, Cochran Armitage test of trend. In bold are the serotypes with significant p-values ($p < 0.05$) after FDR correction. [b]NVT, non-vaccine serotypes. [c]Only serotypes detected in ≥3 isolates in at least one year are shown; the remaining are represented in "Others."

caused by PPV23 serotypes: 8 (from 3.7 to 12.2%, CA p < 0.001), 22F (from 2.4 to 8.2%, CA p < 0.001) and 20 (from 1.0 to 3.7%, CA p = 0.001); and an increase of the NVT 15A (from 1.0 to 3.5%, CA p = 0.002). Even though these changes were statistically supported when analyzing data from 2008 to 2014, in the case of serotypes 19A and 15A, the more disparate values were only detected from 2013 onwards, while for serotype 20, this occurred from 2012 onwards.

Table IIb.2 shows the evolution of IPD serotypes during the study period (2012–2014) according to vaccine serotypes and stratified by age group. Recapitulating what was seen when considering all age groups together (Figure IIb.4), a decrease in the overall proportion of IPD caused by PCV13 serotypes was detected in the three age groups considered; however, only for individuals ≥ 65 years was this statistically supported (Table IIb.2). Moreover, only for this age group was the decrease in the additional serotypes found in both PCV10 and PCV13 (serotypes 1, 5 and 7F) statistically supported after FDR correction (Table IIb.2).

Table IIb.2 Number of isolates responsible for invasive pneumococcal disease in adult patients (≥18 years), according to vaccine serotype groups and age groups, 2012–2014

	Serotype Groups	No. isolates (%)			C. A.[a]
		2012	2013	2014	
18-49 years	PCV7[b]	18 (21.4)	12 (15.0)	8 (11.9)	0.112
	1, 5 and 7F	15 (17.9)	10 (12.5)	8 (11.9)	0.286
	3, 6A and 19A	12 (14.3)	12 (15.0)	11 (16.4)	0.719
	PCV13[c]	45 (53.6)	34 (42.5)	27 (40.3)	0.094
	PPV23 add[d]	26 (31.0)	29 (36.3)	24 (35.8)	0.511
	NVTs[e]	13 (15.5)	17 (21.3)	16 (23.9)	0.191
50-64 years	PCV7[b]	7 (9.2)	14 (13.9)	15 (16.7)	0.164
	1, 5 and 7F	10 (13.2)	6 (5.9)	4 (4.4)	0.037
	3, 6A and 19A	20 (26.3)	19 (18.8)	14 (15.6)	0.087
	PCV13[c]	37 (48.7)	39 (38.6)	33 (36.7)	0.124
	PPV23 add[d]	17 (22.4)	34 (33.7)	37 (41.1)	0.011
	NVTs[e]	22 (28.9)	28 (27.7)	20 (22.2)	0.316
≥65 years	PCV7[b]	30 (12.3)	36 (17.8)	27 (12.3)	0.947
	1, 5 and 7F	20 (8.2)	9 (4.5)	6 (2.7)	**0.008**
	3, 6A and 19A	75 (30.7)	39 (19.3)	50 (22.8)	0.042
	PCV13[c]	125 (51.2)	84 (41.6)	83 (37.9)	0.004
	PPV23 add[d]	67 (27.5)	65 (32.2)	82 (37.4)	0.022
	NVTs	52 (21.3)	53 (26.2)	54 (24.7)	0.384

[a]CA, Cochran Armitage test of trend. In bold are the serotype groups with significant pvalues (p < 0.05) after FDR correction. [b]PCV7, serotypes included in the 7-valent pneumococcal conjugate vaccine. [c]PCV13, serotypes included in the 13-valent pneumococcal conjugate vaccine. [d]PPV23 add, the additional 11 serotypes present in the 23-valent pneumococcal polysaccharide vaccine but absent from the 13-valent pneumococcal conjugate vaccine. [e]NVTs, serotypes not included in any of the currently available pneumococcal vaccines.

When analyzing the evolution of each serotype from 2008 to 2014 stratifying by age group, only serotype 1 decreased in all age groups considered (CA $p < 0.001$ for each, significant after FDR correction), while the increase of serotype 8 was significant only in the two older groups (≥ 50 years) (CA $p < 0.001$ for both, significant after FDR correction), and the changes in serotypes 5, 7F, 19A, 20, and 22F were statistically supported only in individuals ≥ 65 years (CA $p < 0.001$ for serotypes 5 and 7F, CA $p = 0.007$ for serotype 19A, CA $p = 0.003$ for serotype 20 and CA $p = 0.001$ for serotype 22F, all significant after FDR correction).

Antimicrobial Susceptibility

Resistance to the antimicrobials tested is summarized in Table IIb.3. A total of n = 179 isolates (15.4%) were classified as penicillin non-susceptible pneumococci (PNSP): n = 160 (89.4%) presenting low level resistance and n = 19 (10.6%), high level resistance. Considering current CLSI breakpoints for penicillin, n = 12/59 CSF isolates (20.3%) would have been considered resistant and only n = 5/1104 non-CSF isolates (0.5%) would have been considered intermediately resistant. A total of n = 198 isolates (17.0%) were classified as erythromycin resistant pneumococci (ERP). Of these, n = 159 presented the MLS_B phenotype, while the remaining (n = 39, 19.7%) presented the M phenotype. Isolates simultaneously non-susceptible to penicillin and erythromycin (EPNSP) accounted for 10.4% of the collection (n = 121). Antimicrobial resistance did not change significantly between age groups. In 2012–2014, there was a significant decrease in antimicrobial resistance for several antimicrobials — erythromycin resistance decreased from 18.8 to 13.0% (CA $p = 0.034$), clindamycin resistance decreased from 16.1 to 10.4% (CA $p = 0.022$) and tetracycline resistance decreased from 13.4 to 7.7% (CA $p = 0.010$). Although, not statistically supported, there was also a decrease in penicillin non-susceptibility, from 16.8% in 2012 to 13.3% in 2014 (CA $p = 0.174$).

There was some correlation between serotype and antimicrobial resistance (Figures IIb.1–IIb.3). The AW for serotype and PNSP was 0.569 (CI95%: 0.507–0.631) and the AW for serotype and ERP was 0.527 (CI95%: 0.458–0.596). Serotypes 14 and 19A were the most frequent serotypes among PNSP and ERP. Serotype 14 accounted for 35.2% of PNSP and 22.2% of ERP while serotype 19A occurred in 21.2% of PNSP

and 21.2% of ERP. Taken together, PCV7 serotypes accounted for 48.6% of PNSP, 37.9% of ERP and 40.5% of EPNSP. Considering the PCV13 serotypes, these constituted 71.5, 61.1, and 67.8% of PNSP, ERP and EPNSP, respectively. The additional serotypes found in PPV23 but not in PCV13 accounted for only 2.8, 6.6, and 1.7% of PNSP, ERP and EPNSP, respectively. The proportion of resistant isolates was higher among isolates expressing NVTs: 25.7, 32.3, and 30.6% of PNSP, ERP and EPNSP, respectively (Figures IIb.1–IIb.3). The most frequent NVTs among PNSP and ERP were serotypes 6C and 15A, which together accounted for 19.0% of PNSP and 18.2% of ERP (Figure II.3b).

Table IIb.3 Antimicrobial resistance of the isolates responsible for invasive pneumococcal disease in adult patients (≥18 years) in Portugal, 2012–2014.

	No. resistant isolates (%)		
	18-49 years (n=231)	50-64 years (n=267)	≥65 years (n=665)
PEN	40 (17.3)	32 (12.0)	107 (16.1)
MIC_{90}	0.38	0.125	0.25
MIC_{50}	0.016	0.012	0.016
CTX	4 (1.7)	3 (1.1)	6 (0.9)
MIC_{90}	0.25	0.19	0.25
MIC_{50}	0.016	0.016	0.016
LEV	0 (0)	1 (0.4)	6 (0.9)
ERY	34 (14.7)	37 (13.9)	127 (19.1)
CLI	31 (13.4)	30 (11.2)	100 (15.0)
CHL	5 (2.2)	6 (2.2)	8 (1.2)
SXT	30 (13.0)	39 (14.6)	93 (14.0)
TET	23 (10.0)	21 (7.9)	79 (11.9)

PEN, penicillin; CTX, cefotaxime; LEV, levofloxacin; ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; SXT, trimethoprim/sulphamethoxazole; TET, tetracycline. All isolates were susceptible to vancomycin and linezolid

Discussion

The decrease in PCV13 serotypes observed previously (Horácio et al., 2012 and 2013) continued during the present study period resulting in only 38.0% of the isolates collected in 2014 expressing PCV13 serotypes (Figure IIb.4). However, different serotypes underlie the changes in 2008–2011 and 2012–2014.

The timeframes of the decreases seen for serotypes 7F and 19A are consistent with a possible herd protection of childhood vaccination with the most recently introduced PCVs. Similar decreases in serotypes 7F and 19A as causes of adult IPD followed the use of PCV13 in children in the USA (Moore et al., 2015) and in several European countries (Steens et al., 2013; Guevara et al., 2014; Harboe et al., 2014; Waight et al., 2015). Decreases in the incidence of IPD caused by these two serotypes were also documented among children in Portugal (Aguiar et al., 2014). In Portugal the decrease in serotype 7F preceded that of serotype 19A in adult IPD. This could have been attributed to the use of PCV10 in children, since PCV10 includes serotype 7F but not serotype 19A. Moreover, this vaccine was introduced in Portugal months earlier than PCV13. However, in children, serotype 19A decreased as a cause of IPD before an effect of PCV13 was expected and before any decrease in serotype 7F (Aguiar et al., 2014). This points to the importance of other factors besides vaccination in triggering changes in serotype prevalence and suggest that the initial changes seen in serotype 7F IPD in adults are the result of secular trends.

In contrast to these serotypes, there was no overall reduction of serotype 3. These results are concordant with other studies that failed to show a consistent reduction of serotype 3 among adult IPD after the use of PCV13 in children (Steens et al., 2013; Harboe et al., 2014; Moore et al., 2015; Waight et al., 2015) and with a study that demonstrated a low and non-significant effectiveness of PCV13 against serotype 3 IPD in children (Andrews et al., 2014).

The proposed higher efficacy of PCV13 against serotype 19F (Dagan et al., 2013) cannot explain the decrease in proportion of PCV7 serotypes, since serotype 19F was uncommon in our collection and no significant decrease was seen between the two periods (Table IIb.1).The reduction of the overall proportion of IPD caused by PCV7 serotypes was instead related with decreases in serotypes 4, 9V and 14 (Table IIb.1). Among these, serotype 4 exhibited the most significant decrease. Since the most

significant decrease of serotype 14 IPD was detected in 2014, it remains uncertain if it will be sustained in the following years. Serotype 14 has been the most frequent PCV7 serotype causing adult IPD in Portugal, both before and after PCV7 use in children. This could be associated with particular characteristics of the highly successful and resistant clone Spain14-ST156, to which this serotype was found to be associated (Horácio et al., 2016a). High antimicrobial consumption in our country could also contribute significantly to maintain resistant clones such as this one in circulation.

The non-PCV serotypes that increased the most since the late-post PCV7 period were those found in PPV23, especially serotypes 8, 22F, and 20 (ranked by frequency); but also the non-PPV23 serotype 15A (Table IIb.1). Serotypes 15A and 22F were found in carriage in adults in Portugal (Almeida et al., 2014), while serotypes 8 and 20 were not found in carriage in adults and were shown to have a high invasive disease potential (Sá-Leão et al., 2011). Serotype 8 was the second most frequent cause of IPD during the current study period and in 2013 and 2014 was the most frequent cause of IPD among younger adults (18–49 years). Serotype 8 increased in importance as a cause of IPD in other countries, being the most frequent cause of IPD in patients aged > 5 years in England and Wales after the introduction of PCV13 (Waight et al., 2015) and also important in adult IPD elsewhere (Guevara et al., 2014; Regev-Yochay et al., 2015). Serotype 22F became the second most frequent cause of IPD in adults aged ≥ 65 years in 2013 and 2014. In the USA, this serotype was the most common cause of adult IPD in the post-PCV13 period (Moore et al., 2015). An increase of serotype 22F after PCV13 use was also reported in Canada (Demczuk et al., 2013) and in some European countries (Steens et al., 2013; Lepoutre et al., 2015). Serotype 20 increased more modestly and only among individuals aged ≥ 65 years. An increase of this serotype was also noted in Canada, although mostly among individuals aged 15–49 years (Demczuk et al., 2013). Taken together, these observations indicate that, although there may be some regional differences, there are serotypes that seem to be consistently emerging in different geographic locations in the post-PCV13 period. These may reflect circulating serotypes in asymptomatic carriers but also serotypes with an enhanced invasive disease potential.

In 2014, the last year of the study, serotype 15A surpassed serotype 19A and 14 to become the most frequent serotype among ERP and was the second most frequent

serotype among PNSP behind serotype 14. The overall decreases observed in PNSP and ERP were not only due to decreases in the total number of isolates expressing serotypes 14 and 19A, which were not compensated by the increase in serotype 15A (Table IIb.1), but also to an unexpected decrease in the proportion of resistant isolates within serotypes 14 and 19A. While 72% of serotype 14 and 64% of serotype 19A were ERP in 2012, only 44% of serotype 14 and 33% of serotype 19A were ERP in 2014 ($p = 0.071$ and $p = 0.031$, respectively). Similarly, there was a decrease in the proportion of PNSP among serotype 19A, from 59% in 2012 to 24% in 2014 ($p = 0.014$).

Our surveillance system is exclusively laboratory based and lacks compliance audits, so our study was not designed to estimate the incidence of adult IPD. However, we did note a slight decrease in the number of isolates sent to us in 2013 and 2014 (Figure IIb.4). This could reflect a net reduction of adult IPD following PCV13 use in children, as reported by others (Guevara et al., 2014; Harboe et al., 2014; Lepoutre et al., 2015; Moore et al., 2015; Regev-Yochay et al., 2015) and seen with IPD in children in Portugal (Aguiar et al., 2014). Alternatively, this could reflect lower reporting by participating laboratories. We also noted a marked decrease in the number of isolates recovered from younger patients relative to either of the older age groups when comparing 2009–2011 to 2012–2014 ($p < 0.001$) (Figure IIb.1) (Horácio et al., 2013). Even if the decrease in number of isolates is attributed to lower reporting, we have no reason to believe that this would affect preferentially a particular age group. We also have no indication of changes in clinical practice (such as blood culturing practices), which could influence these results. We therefore believe that the most likely explanation is a true reduction in incidence of IPD in 18–49 years old individuals, in agreement with a study from the UK that found that this group was the one where the decrease in IPD incidence was more pronounced and followed more closely PCV13 use in children (Waight et al., 2015).

As discussed above, our study was not designed to allow the estimate of the incidence of IPD and it therefore does not evaluate potential changes in incidence with time. Specifically, although we include the majority of medical centers in Portugal our surveillance is not comprehensive and we did not perform audits to ensure that participating centers reported all cases, namely we did not include cases for which no viable pneumococcal isolate was received for characterization. However,

the design based on the reporting of all isolates causing IPD within the surveillance network, the large number of isolates studied, the wide coverage of the country by the network and the stable number of reporting centers, guarantees that the data accurately represents IPD in Portugal and can be used to evaluate changes in the relative importance of the different serotypes.

In spite of relatively modest vaccine coverage (63% in 2012), there were major changes in the serotype distribution of the pneumococcal population responsible for adult IPD in Portugal following the use of PCVs in children consistent with herd protection. These changes have contributed also to significant reductions in antimicrobial resistance. The recent inclusion of PCV13 in the NIP for children in Portugal may have an even greater impact on IPD in adults. This remarkable effect of PCVs in protecting non-vaccinated individuals may question the need of using PCV13 directly in vaccinating adults. Still, data from 2014 indicates that the overall proportion of adult IPD caused by PCV13 serotypes remained significant (38%) and that isolates expressing PPV23 serotypes accounted for 75% of all IPD. Taken together this suggests a key role of vaccination in any effective management strategy of IPD.

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Author Contributions

JM and MR: Conceived and designed the experiments. PGSSI: Collected data. AH, CS, and JL: Performed the experiments. AH, JM, and MR: Analyzed the data. All authors contributed to the writing of the manuscript and approved the version to be submitted.

Supporting Information

Table IIb.S1: Capsular types of the isolates recovered from CSF in adult patients (≥18 yrs), Portugal, 2012-2014.

	No. Isolates		
Serotype	**CSF**	**non-CSF**	**OR ($CI_{95\%}$)**
3	12	149	1.20 (0.56-2.30)
19F	**6**	**21**	**4.45 (1.41-12.04)**
11A	4	45	1.30 (0.33-3.76)
23B	**4**	**8**	**7.60 (1.62-29.47)**
8	3	120	0.33 (0.06-1.03)
16F	3	20	2.22 (0.41-7.82)
24F	3	20	2.22 (0.41-7.82)
Others	24	466	0.56 (0.32-0.99)

CHAPTER III: INVASIVE PNEUMOCOCCAL DISEASE – CLONES AND PILI

RATIONALE

In the studies presented in the previous chapter we found significant changes in the serotype distribution of isolates responsible for adult IPD in Portugal, both in the post-PCV7 period and in the early post-PCV10/PCV13 period. If there were changes in the serotypes associated with adult IPD, there must have been changes in the genetic lineages associated with adult IPD also. In the study presented in this chapter (Horácio et al., 2016a) we aimed to identify the genetic lineages responsible for adult IPD in Portugal. The period analyzed (2008-2011) included the late-post PCV7 period and the first years of PCV10/PCV13 use in children. We also evaluated the prevalence of pilus islands in this collection of isolates.

The study composing this chapter continues two other published studies from the laboratory – the study of Serrano et al. (2005) and the study of Aguiar et al. (2008b). The study of Serrano et al. (2005) evaluated the genetic lineages of invasive pneumococci before the widespread use of PCV7 in children (1999-2002). They detected a stable clonal structure and found most lineages were represented in all years of the study period. The clonal diversity within each serotype varied, with some serotypes being highly diverse in their clonal structure, while others were represented by only few genetic lineages. Representatives of some of the internationally disseminated clones of that time were detected in the collection. In the study of Aguiar et al. (2008b) the prevalence of PI-1 was determined. They found that only a small proportion of isolates (27%) were positive for the *rlrA* gene and that the presence of PI-1 was a clonal property of pneumococci, despite a strong correlation with serotype.

The following study was performed by Andreia N. Horácio, Catarina Silva-Costa, Jorge Diamantino-Miranda, Joana P. Lopes, Mário Ramirez, José Melo-Cristino and the Portuguese Group for the Study of Streptococcal Infections. Andreia N. Horácio performed a significant part of the experimental work, analyzed the data and wrote the first versions of the manuscript. Part of the 2008 and 2009 data were previously included in the studies of two master thesis (one of them from Andreia N. Horácio and the other from Jorge Diamantino-Miranda).

POPULATION STRUCTURE OF *STREPTOCOCCUS PNEUMONIAE* CAUSING INVASIVE DISEASE IN ADULTS IN PORTUGAL BEFORE PCV13 AVAILABILITY FOR ADULTS: 2008-2011[1]

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Abstract

Among the 1660 isolates recovered from invasive pneumococcal disease (IPD) in adults (≥ 18yrs) in 2008–2011, a random sample of ≥ 50% of each serotype (n = 871) was chosen for MLST analysis and evaluation for the presence and type of pilus islands (PIs). The genetic diversity was high with 206 different sequence types (STs) detected, but it varied significantly between serotypes. The different STs represented 80 clonal complexes (CCs) according to goeBURST with the six more frequent accounting for more than half (50.6%) of the isolates — CC156 (serotypes 14, 9V and 23F), CC191 (serotype 7F), CC180 (serotype 3), CC306 (serotype 1), CC62 (serotypes 8 and 11A) and CC230 (serotype 19A). Most of the isolates (n = 587, 67.3%) were related to 29 Pneumococcal Molecular Epidemiology Network recognized clones. The overall proportion of isolates positive for any of the PIs was small (31.9%) and declined gradually during the study period (26.6% in 2011), mostly due to the significant decline of serotype 1 which is associated with PI-2. The changes in serotypes that occurred in adult IPD after the introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) for children were mostly due to the expansion of previously circulating clones, while capsular switching was infrequent and not related to vaccine use. The reduction of IPD caused by PCV7 serotypes in the years following PCV7 implementation did not result in a decline of antimicrobial resistance in part due to the selection of resistant genotypes among serotypes 14 and 19A.

[1] A *facsimile* of this publication can be found at the "Publications" section of this thesis.

Introduction

The 7-valent conjugate vaccine (PCV7) was available for children through the private sector in Portugal from 2001 onwards until it was replaced in the beginning of 2010 by the 13-valent conjugate vaccine (PCV13). In 2012, PCV13 received approval for use also in adults > 50 years of age with an extension being made to all ages in 2013. Additionally, PCV13 entered the Portuguese National Immunization Program (NIP) in June 2015 for children born from January 2015 onwards. Two other vaccines, the 23-valent pneumococcal polysaccharide vaccine (PPV23) and the 10-valent conjugate vaccine (PCV10), have also been available in Portugal since 1996 and 2009, respectively, but with a low uptake (Horácio et al., 2012).

Among the more than 90 different pneumococcal serotypes identified, only a few cause the majority of IPD. While for some serotypes the capsular polysaccharide is the dominant determinant of invasiveness, for others distinct genotypes show important differences in invasiveness (Sá-Leão et al., 2011). Additionally, there are other features that are strongly associated with genotype independently of serotype, such as antimicrobial susceptibility and the presence and type of pilus islands (Aguiar et al., 2008b and 2010b). With the availability of pneumococcal conjugate vaccines that efficiently target particular serotypes, important changes have been reported regarding not only serotype but also genotype distributions of pneumococci causing IPD (Beall et al., 2006; Aguiar et al., 2010a; Bettinger et al., 2010; Pilishvili et al., 2010; Rodenburg et al., 2010). Interestingly, while non-vaccine serotypes have emerged as a cause of IPD, in some cases distinct clones expressing the same serotype have risen in frequency in different geographic regions (Pai et al., 2005; Serrano et al., 2005).

While numerous studies have addressed the serotype distribution of IPD, information regarding the clonal composition of pneumococcal populations has been scarcer. In a previous study we defined the clonal composition of pneumococci causing IPD in both children and adults in the pre-PCV7 period (Serrano et al., 2005). In a subsequent study we documented major changes in the potential coverage of PCV13 starting in 2009, due to decreases in prevalence of serotypes 1 and 5 (Horácio et al., 2013). In the present study we aimed to characterize the clonal composition of pneumococci causing adult IPD in Portugal between 2008 and 2011, a period

characterized by extensive use of PCV7 and the adoption of PCV13 in children and prior to the use of PCV13 in adults.

Materials and Methods

Bacterial Isolates

The isolates included in this study were recovered from adult patients (≥ 18 yrs) with invasive pneumococcal disease between 2008 and 2011 and were characterized in previous studies regarding serotype distribution and antimicrobial susceptibility (Horácio et al., 2012 and 2013). A case of invasive disease was defined by the recovery of pneumococci from a normally sterile source, such as blood or cerebral spinal fluid (CSF). Serotypes were grouped into conjugate vaccine serotypes, i.e., those included in PCV13 (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 19A, 23F) that comprise all serotypes found in lower valency vaccines (PCV7: 4, 6B, 9V, 14, 18C, 19F, 23F; and PCV10: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F), those included in PPV23 (all serotypes included in PCV13 except 6A and serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F), and non-vaccine serotypes (NVT). The isolates that were not typable with any of the complete set of sera available from the Staten Serum Institute (Copenhagen, Denmark) were considered non-typable (NT). Given the high frequency of spontaneous switching between serotypes 15B and 15C we opted to include strains with these serotypes into a single group. Due to the difficulty in distinguishing a set of isolates that were positive for both serotypes 25A and 38 we opted to include strains with these serotypes into a single group.

From a total of 1660 isolates recovered, a random sample of ≥ 50% of the isolates (n = 871) from each serotype and from each year was chosen to be characterized by MLST and tested for the presence of the pilus islands. Briefly, among the 1660 isolates, there were 52 different serotypes, with the 10 most frequent being serotypes 3 (13.0%), 7F (10.0%), 19A (9.8%), 1 (8.5%), 14 (8.1%), 8 (5.7%), 22F (3.9%), 4 (3.2%), 9N (2.8%) and 11A (2.8%). However, the 10 most frequent serotypes were different in each of the age groups. In the 18–49 yr olds (n = 472) these were serotypes 1 (14.0%), 7F (11%), 8 (9.1%), 14 (8.7%), 3 (7.6%), 19A (6.1%), 9N (4.2%), 4 (3.8%), 22F (3.2%), 11A (2.8%). In the 50–64 yr olds (n = 358) these were serotypes 3 (12%), 19A (10.3%), 1 (9.5%), 7F (9.2%), 14 (6.1%), 4 (5.0%), 8 (4.5%), 11A (3.4%), 22F (3.4%), 9V (2.8%). In the 65 yr olds (n = 830) these were serotypes 3 (15.8%), 19A (11.6%), 7F (10.2%), 14 (8.7%), 1 (4.9%), 22F (4.6%), 8 (4.2%), 6C (3.0%), 11A (2.5%), 9N (2.3%). Overall, the

proportions of PCV7, PCV13 and PPV23 serotypes were 18.4%, 61.9% and 79.4%, respectively. Non-susceptibility to penicillin, defined as either intermediate level penicillin resistance (MIC 0.12–1.0 μg/ml) or high level resistance (MIC ≥ 2.0 μg/ml) as discussed previously (Horácio et al., 2012 and 2013), was found in 330 isolates (19.9%), while 315 isolates (19.0%) were resistant to erythromycin. The age and sex of the patients and the source of the isolates randomly chosen for further study was similar to that of the 1660 isolates. In the genotyped group the age distribution was as follows: 28.5% of the isolates were from individuals 18–49 yrs, 21.2% from 50–64 yrs and 50.3% from ≥ 65 yrs. The majority of the isolates were collected from blood (87.9%), 8.4% from CSF, 2.5% from pleural fluid and 1.2% from other normally sterile sources.

MLST

MLST was performed as described previously (Enright and Spratt, 1998). The DNA sequences were analyzed using Bionumerics software (Applied-Maths, Sint-Martens-Laten, Belgium) and the alleles and sequence types were assigned according to the pneumococcal MLST database available at http://pubmlst.org/spneumoniae/. The goeBURST algorithm (Francisco et al., 2009) implemented in the PHYLOViZ software (Francisco et al., 2012) was used to establish relationships between STs. Clonal complexes were defined at the single-locus-variant (SLV) and double-locus-variant (DLV) levels.

Detection of Pilus Islands

The presence of pilus islets (PI) was evaluated by PCR. Briefly, for PI-1 in the absence of the pilus islet, a product of 1-3Kb was expected using primers PFL-up and P-dn flanking the islet (Aguiar et al., 2008b). In strains yielding no PCR product, the *rlrA* gene was detected using primers RLRA-up and RLRA-dn. A similar approach was followed to detect the presence of PI-2 (Aguiar et al., 2012).

Statistical Analysis

Sample diversity was evaluated using the Simpson's index of diversity (SID) and the respective 95% confidence intervals (CI95%) (Carriço et al., 2006). To compare

two sets of partitions the Adjusted Wallace (AW) coefficients were calculated (Severiano et al., 2011) using the online tool available at http://www.comparingpartitions.info. Differences were evaluated by the Fisher exact test with the false discovery rate (FDR) correction for multiple testing (Benjamini and Hochberg, 1995) and the Cochran-Armitage test was used for trends. A $p < 0.05$ was considered significant for all tests.

Results

Sequence Type Distribution and Relationship with Serotype

The 871 isolates analyzed by MLST presented 206 different STs (SID = 0.971, CI95%: 0.967–0.976) grouping into 80 CCs (SID = 0.948, CI95%: 0.942–0.953) according to goeBURST analysis, when including all STs deposited in the database. The 14 most frequent STs, which accounted for more than half of the genotyped isolates (50.6%) were, in decreasing order, ST191 (9.9%), ST306 (7.0%), ST180 (6.9%), ST53 (4.5%), ST156 (4.0%), ST276 (3.6%), ST433 (3.2%), ST66 (2.8%), ST408 (1.7%), ST232 (1.7%), ST260 (1.5%), ST143 (1.4%), ST179 (1.3%) and ST289 (1.3%).

Twenty new allelic combinations and 19 new alleles were identified. The new allelic combinations were identified as STs: 6176, 6177, 6180, 6181, 6182, 6973, 8866, 9955, 9956, 9957, 9958, 9960, 9963, 9966, 9969, 9970, 9971, 9979, 9982 and 9986. The novel alleles identified were designated 200, 307 and 309 for *aroE*, 429, 636 and 637 for *ddl*, 294, 295, 428 and 430 for *gdh*, 437 and 438 for *gki*, 273 for *recP* and 588, 589, 590, 592, 593 and 605 for *xpt*.

There was a strong correlation between CC and the vaccine serotype groups (AW = 0.810, CI95%: 0.763–0.857), with the six most prevalent CCs being mainly composed of isolates presenting vaccine serotypes (95.5%). Table III.1 shows the age distribution and serotypes of the most frequent STs found in the 22 major CCs (n ≥ 10 isolates), together accounting for 83.7% of the genotyped isolates. The major CC (CC156, n = 101) included mostly isolates expressing PCV7 serotypes, namely 14, 9V and 23F, while four of the remaining five most frequent CCs were mainly composed of isolates presenting the additional serotypes found in PCV13 (mainly 7F, 3, 1 and 19A). The other most frequent lineage, CC62, consisted mostly of isolates expressing serotypes included only in PPV23 (serotypes 8 and 11A). The age distribution and serotypes of the STs found in CCs with < 10 isolates are shown in III.S1 Table.

Table III.1 Age distribution and the serotypes of the most frequent STs found in the 22 major CCs (n≥10 isolates) identified by goeBURST.

CC (n)	ST	Total	no. of isolates			Dominant serotype (n)	Other serotypes
			[18-49]	[50-64]	>=65		
156 (101)	156	35	13	5	17	14 (31)	9V (3), 10A (1)
	143	12	2	2	8	14 (12)	-
	338	10	3	1	6	23F (7)	23A (2), 19F (1)
	162	6	2	1	3	9V (4)	19F (1), 24A (1)
	2944	5	0	3	2	14 (5)	-
	Others[a]	33	8	6	19	9V (10)	14 (8), 6B (5), 6C (3), 23F (3), 35F (2), 17F (1), 17A (1)
191 (88)	191	86	30	14	39	7F (83)	NT[c] (2), 7A (1)
	Others[a]	2	0	2	0	7F (2)	-
180 (68)	180	60	8	17	35	3 (60)	-
	Others[a]	8	2	1	5	3 (8)	-
306 (68)	306	61	26	14	21	1 (61)	-
	350	5	1	2	2	1(5)	-
	Others[a]	2	2	0	0	1(2)	-
62 (67)	53	39	15	8	16	8 (37)	NT (2)
	408	15	6	4	5	11A (14)	11C (1)
	62	7	1	2	4	11A (7)	-
	Others[a]	6	3	0	3	8 (2), 11A (2)	18C (1), 22F (1)
230 (47)	276	31	3	7	21	19A (31)	-
	230	6	1	1	4	24F (4)	19A (2)
	Others[a]	10	4	3	3	19A (8)	10A (1), 24F (1)
81 (30)	66	24	12	3	9	9N (23)	NT (1)
	Others[a]	6	1	0	5	24F (4)	4 (2)
433 (29)	433	28	4	5	19	22F (28)	-
	Others[a]	1	0	0	1	22F (1)	-
439 (25)	439	7	0	3	4	23B (7)	-
	42	5	1	3	1	23A (4)	6A (1)
	Others[a]	13	3	2	8	23A (7)	23B (3), 23F (3)
15 (24)	9	8	3	0	5	14 (8)	
	1201	7	3	0	4	19A (4)	7C (3)
	Others[a]	9	1	2	6	14 (5)	34 (2), 6B (1), 7C (1)
177 (24)	179	11	4	4	3	19F (11)	-
	Others[a]	13	3	5	5	19A (5)	19F (3), 21 (3), 15A (1), 15 B/C (1)
199 (21)	416	8	1	2	5	19A (8)	-
	411	7	1	2	4	15B/C (7)	-
	199	6	2	2	2	19A (3)	15B/C (2), 18C (1)
378 (19)	232	15	3	5	7	3 (15)	-
	Others[a]	4	2	0	2	3 (4)	-
113 (16)	123	5	1	0	4	17F (5)	-
	1766	5	2	0	3	31 (5)	-
	Others[a]	6	1	2	3	22F (3)	17F (1), 18C (1), 31 (1)
460 (16)	97	10	2	3	5	10A (10)	-
	Others[a]	6	3	1	2	6A (4)	10A (1), 35F (1)
260 (15)	260	13	2	3	8	3 (13)	-
	Others[a]	2	1	0	1	3 (2)	-
218 (13)	218	10	3	2	5	12B (10)	-
	Others[a]	3	1	1	1	12B (2)	12F (1)
289 (13)	289	11	5	2	4	5 (11)	-
	Others[a]	2	1	0	1	5 (2)	-

Table III.1 (continued)

CC (n)	ST	Total	no. of isolates			Dominant serotype (n)	Other serotypes
			[18-49]	[50-64]	>=65		
30 (11)	30	10	2	2	6	16F (10)	-
	Others[a]	1	0	0	1	16F (1)	-
63 (11)	63	8	3	0	5	15A (7)	15F (1)
	Others[a]	3	0	1	2	3 (1), 7F (1), 15A (1)	-
315 (11)	386	7	1	1	5	6C (6)	6B (1)
	Others[a]	4	0	1	3	6C (3)	6B (1)
404 (10)	404	9	5	0	4	8 (9)	-
	Others[a]	1	1	0	0	8 (1)	-

[a] Sequence types that accounted for less than 5 isolates each were grouped together in "Others". [b] NT-non typable.

Figure III.1 shows the STs expressing each of the 13 serotypes included in PCV13 and Figure III.2 the STs expressing each of the 10 most frequent serotypes found among those not included in any of the conjugate vaccines. The STs found in the remaining serotypes are indicated in III.S2 Table. The genetic diversity varied greatly with serotype, with serotypes, 4, 6A, 6B, 9V, 18C, 19A, 20 and 23A being highly diverse (SID > 0.8) and serotypes 1, 5, 7F, 9N and 22F displaying very limited diversity (SID < 0.3). In general, there was a predominance of high genetic diversity among PCV13 serotypes and low genetic diversity among the 10 most frequent non-PCV13 serotypes. For serotypes 9V, 14 and 23A, the wide variety of STs did not result in a high diversity of CCs, with a maximum of two CCs being detected in each. The genetic diversity of each serotype was independent of the serotype's frequency. Examples of this are the low frequency serotypes 6B and 18C that presented a high genetic diversity and no dominant ST.

A total of 587 isolates (67.3%) presented STs related to 29 of the 43 clones recognized by the Pneumococcal Molecular Epidemiology Network (PMEN) (McGee et al., 2001), sharing at least five MLST alleles with these clones (357 isolates had the same ST, 133 were SLVs and 97 were DLVs). When considering these isolates the predominant clones were Netherlands7F-191 (n = 88), Spain9V-156 (n = 71), Netherlands3-180 (n = 68), Netherlands8-53 (n = 63), Sweden1-306 (n = 63), Denmark14-230 (n = 47), Tennessee14-67 (n = 24), Tennessee23F-37 (n = 24) and Netherlands15B-199 (n = 21) (Figs III.1 and III.2 and III.S2 Table). Additionally, another 63 isolates were included in the same CCs of other four PMEN clones.

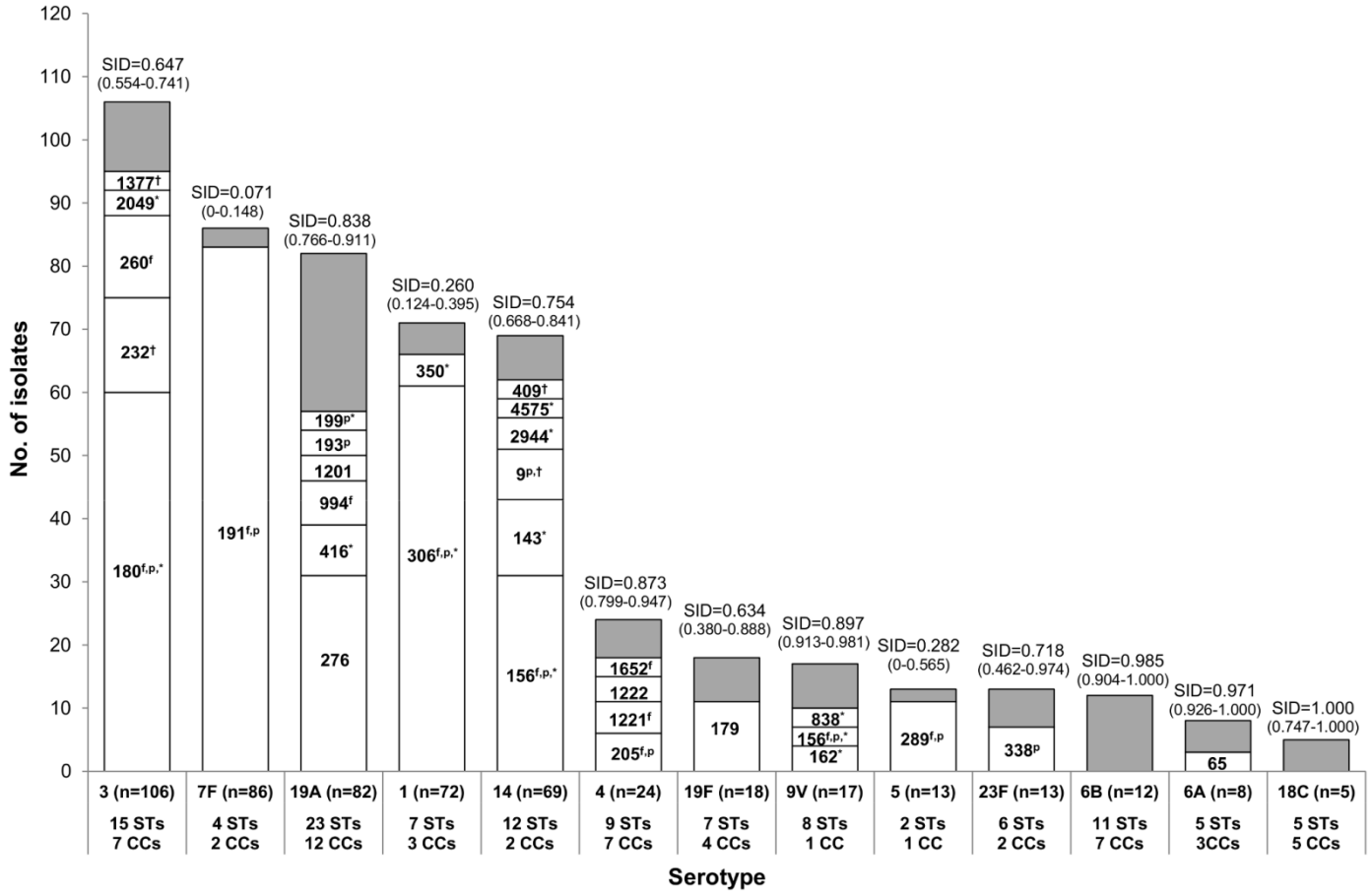

Figure III.1 Distribution of STs according to serotype of the isolates causing adult IPD in 2008–2011 and expressing serotypes included in the conjugate vaccines. The STs that were considered by goeBURST as founders of a CC are indicated by "f". The STs that matched the STs of PMEN clones are indicated by "p". Marked either with "*" or "†" are STs belonging to the same CC in each serotype. The respective SID values are indicated on top of the bars and in parenthesis are the respective confidence intervals. In grey are represented the isolates included in STs with < 3 isolates. These were: serotype 4 – ST801 (n = 2) and STs 244, 246, 259 and 1866 (n = 1, each); serotype 6B – ST 176 (n = 2), STs 138, 273, 386, 473, 1518, 6175, 9957, 9970, 9986 and 10051 (n = 1, each); serotype 9V –STs 280 and 10044 (n = 2, each) and STs 239, 1762 and 10054 (n = 1, each); serotype 14 – ST15 (n=2) and STs 2511, 2616, 4573, 4576 and 10041 (n = 1, each); serotype 18C – STs 102, 113, 199, 1233 and 10033 (n = 1, each); serotype 19F –ST 177 (n = 2), STs 89, 162, 271, 338 and 391 (n = 1, each); serotype 23F – ST 10039 (n = 2) and STs 1135 and 9579 (n = 1, each); serotype 1 –STs 217, 228, 1233, 3081 and 4578 (n = 1, each); serotype 3 – ST 1220 (n=2) and STs 505, 1230, 6014, 9162 and 10038 (n = 1, each); serotype 5 – STs 280 and 10044 (n = 2, each), STs 239, 1762 and 10054 (n = 1, each); serotype 6A –ST 1876 (n=2) and STs 42, 460 and 10055 (n = 1, each); serotype 7F – STs 1062, 1589 and 3130 (n = 1, each) and serotype 19A – STs 230, 242, 320, 2013 and 6174 (n = 2, each) and STs 241, 878, 2102, 2669, 2732, 4197, 4847, 6178, 6973, 9963 and 10042 (n = 1, each).

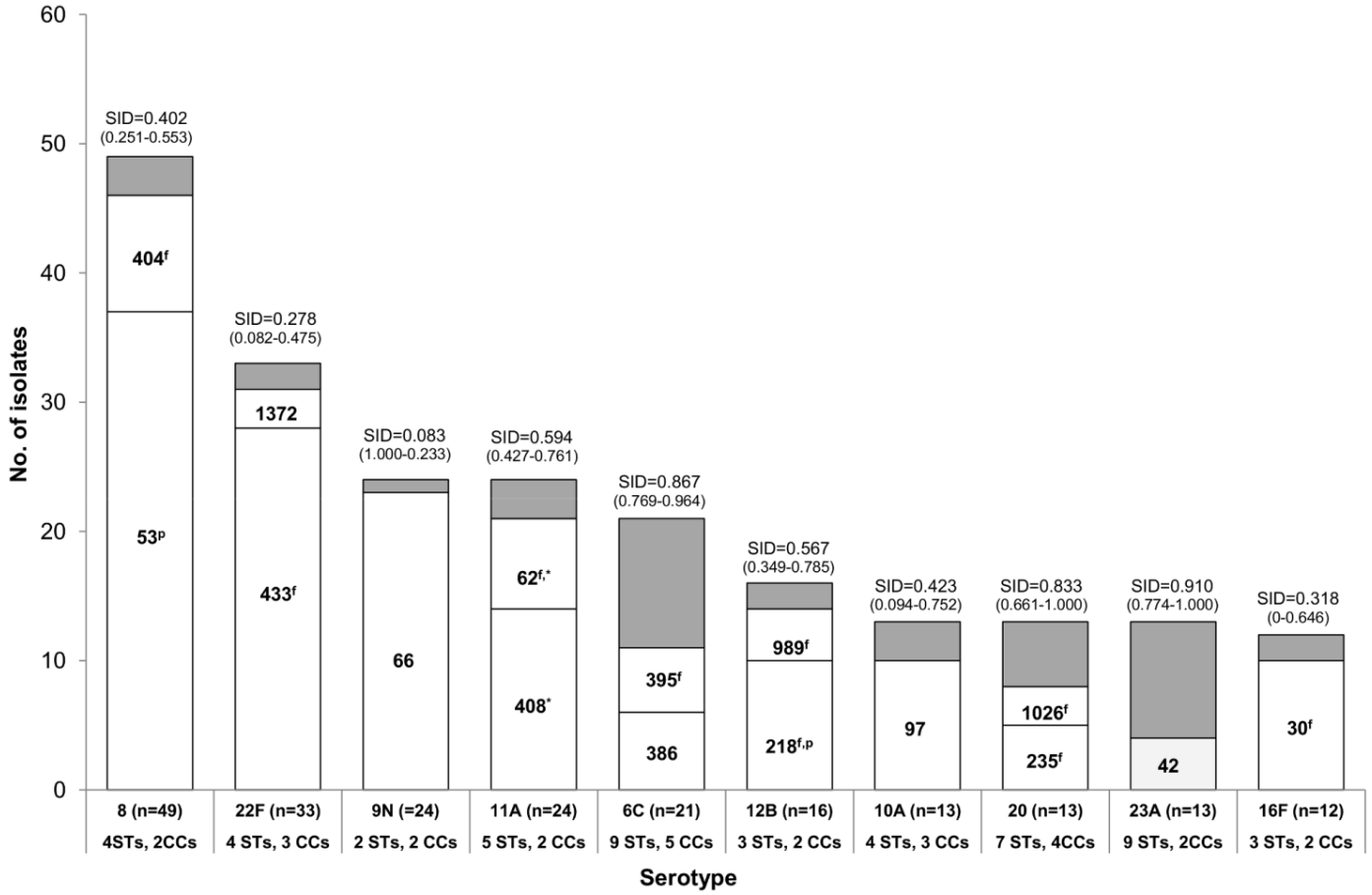

Figure III.2 Distribution of STs according to serotype of the isolates causing adult IPD in 2008–2011 and expressing the 10 most frequent serotypes not included in any of the conjugate vaccines. The respective SID values are shown on top of the bars and in parenthesis are the respective confidence intervals. In grey are represented the isolates included in STs with < 3 isolates. These were: serotype 8 – ST 1012 (n = 2) and ST 9969 (n = 1); serotype 22F – STs 10053 and 10220 (n = 1, each); serotype 9N – ST 3982 (n = 1); serotype 11A – STs 9955, 9960 and 10052 (n = 1, each); serotype 6C – STs 1150, 1692 and 3396 (n = 2, each) and STs 1390, 1715, 2667 and 4310 (n = 1, each); serotype 12B – ST 6180 (n = 2); serotype 10A – STs 156, 816 and 3135 (n = 1, each); serotype 20 – STs 1483, 1871, 7221, 9958 and 10047 (n = 1, each); serotype 23A – ST 338 (n = 2) and STs 190, 311, 438, 6177, 7960, 8866 and 10048 (n = 1, each); serotype 16F – STs 570 and 5902 (n = 1, each).

The correlation between ST and serotype was high (AW = 0.942, CI95%: 0.912–0.973), but there were STs that presented more than one serotype (Table III.1 and III.S1 and III.S2 Tables). The serotype distribution along the studied years for the STs expressing more than one serotype is shown in Table III.2.

Variation of STs with Time

When analyzing the evolution of STs between 2008 and 2011 we identified some fluctuations, although the majority reflected changes in serotype prevalence occurring in this period. However, while for ST306 (serotype 1) there was a decline, significant after correcting for multiple testing (from 11.0% to 2.8%, Cochran-Armitage test of trend p = 0.014), for the other STs the changes were only significant before FDR correction. The STs for which there was a significant p-value in the Cochran-Armitage test for trends but unsupported after FDR correction were: ST53 (serotype 8), that increased from 3.3% to 6.5% (p = 0.043); ST289 (serotype 5), that accounted for 2.4% of IPD in 2008 and 0% in 2011 (p = 0.020); ST717 (serotypes 33A and 33F) that increased from 0% to 1.4% (p = 0.048); and STs 193 (serotype 19A) and 409 (serotype 14) that were only detected in 2008 (1.9% and 1.4%, respectively; p = 0.001 and p = 0.020, respectively). Regarding changes in CCs with time, these reflected the changes identified in STs, with only CC306 declining significantly after FDR correction (from 12.9% to 2.8%, Cochran-Armitage test of trend p = 0.001).

Relationship of STs with Patient Age and Isolate Source

When grouping the isolates according to the three patient age groups – 18–49 yrs, 50–64 yrs and ≥ 65 yrs — only CC5902 showed a statistically significant association with age. The seven isolates belonging to this CC were all recovered from individuals with 18–49 yrs (p = 0.011, significant after FDR correction, III.S1 Table).

When testing for associations between STs and CCs and isolate source, the only significant association found was between CC460 and CSF, with 6 out of 16 isolates being collected from CSF (p = 0.012, significant after FDR correction).

Table III.2 Serotype distribution for the STs expressing more than one serotype between 2008–2011.

	Serotype (n)			
ST[a] (n)	**2008**	**2009**	**2010**	**2011**
156 (35)	14 (6), 9V (1)	14 (12), 9V (1)	14 (7), 10A (1)	14 (6), 9V (1)
338 (10)	-	23F (3)	23F (2), 23A (1), 19F (1)	23F (2), 23A (1)
717 (9)	-	33A (1)	33A (4), 33F (1)	33A (2), 3 (1)
63 (8)	15A (2)	15A (3)	15A (2), 15F (1)	-
386 (7)	-	6C (2)	6C (2)	6C (2), 6B (1)
1201 (7)	19A (2), 7C (1)	19A (1), 7C (1)	19A (1), 7C (1)	-
162 (6)	9V (3), 19F (1)	-	9V (1)	24A (1)
199 (6)	15B/C (1)	15B/C (1), 19A (2), 18C (1)	-	19A (1)
230 (6)	24F (2)	-	24F (2), 19A (1)	19A (1)
42 (5)	6A (1)	23A (2)	23A (2)	-
241 (5)	18A (3)	-	18A (1), 19A (1)	-

[a] Only the sequence types that presented ≥ 5 isolates are shown.

Presence of Pilus Islands

A total of 278 isolates, representing 31.9% of the genotyped collection, carried at least one PI. Among these, 107 (38.5%) had only PI-1, 165 (59.4%) only PI-2 and 6 (2.2%) presented the two PIs simultaneously. While the proportion of PI-1 positive isolates remained stable between 2008 and 2011 (from 10.0% to 11.6%, Cochran-Armitage test of trend p = 0.857), there was a significant decline of PI-2 carrying isolates (from 24.8% to 15.8%, Cochran-Armitage test of trend (p = 0.007). This also resulted in an overall increase in the proportion of isolates lacking any of the pilus islands, from 63.8% in 2008 to 72.6% in 2011 (p = 0.013).

The presence and variants of the PIs were more strongly associated with ST (AW = 0.950, CI95%: 0.933–0.967) than with serotype (AW = 0.711, CI95%: 0.651–0.771). The STs that were significantly associated with PI-1 and PI-2 are shown in Table III.3. All isolates included in CC320 (n = 3) and CC2669 (n = 3) presented the two PIs simultaneously.

Among the 105 isolates presenting only PI-1, 87.9% expressed PCV7 serotypes, namely serotypes 14 (n = 49), 4 (n = 15), 19F (n = 13), 9V (n = 11) and 6B (n = 6). The remaining isolates were from serotypes 19A (n = 9) and 7F, 24A and 35B (n = 1, each). PI-2 positive isolates were from serotypes 7F (n = 85), 1 (n = 68), 11A (5), 19A (n = 2), 3, 7A and 31 (n = 1, each) and NT (n = 2). The isolates presenting simultaneously the two types of PIs were from serotypes 19A (n = 5) and 19F (n = 1).

Table III.3 Sequence types that were associated with pilus island 1 (PI-1) and pilus island 2 (PI-2).

Type of Pilus	ST	Yes	No	OR[a] (95% CI)	p-value[b]
Pilus 1	156	26	9	24.68 (10.79-61.89)	<0.001
	143	12	0	Inf (20.32-Inf)	<0.001
	179	10	1	72.89 (10.18-3133.46)	<0.001
	416	8	0	Inf (12.04-Inf)	<0.001
	162	6	0	Inf (8.16-Inf)	<0.001
	205	6	0	Inf (8.16-Inf)	<0.001
	2944	5	0	Inf (6.30-Inf)	<0.001
	1221	5	0	Inf (6.30-Inf)	<0.001
	4575	3	0	Inf (2.8-Inf)	0.002
	838	3	0	Inf (2.8-Inf)	0.002
	191	0	86	0 (0-0.27)	<0.001
	306	0	61	0 (0-0.39)	<0.001
	180	0	60	0 (0-0.40)	<0.001
Pilus 2	191	86	0	Inf (181.20-Inf)	<0.001
	306	61	0	Inf (99.04-Inf)	<0.001
	350	5	0	Inf (3.81-Inf)	<0.001
	180	0	60	0 (0-0.24)	<0.001
	53	0	39	0 (0-0.39)	<0.001
	156	0	35	0 (0-0.44)	<0.001

[a]OR — Odds ratio. [b]Only significant values after FDR correction are shown.

No associations between isolate source and type of PI were detected. Still, there was a low proportion of PI-2 positive isolates among isolates recovered from the CSF, with only 6 of the 73 CSF isolates presenting PI-2, and while 7 of the 15 isolates recovered from pleural fluid carried PI-2, none carried PI-1.

Antimicrobial Resistance

Similarly to pilus islands, resistance to antimicrobials was more strongly associated with ST than with serotype. The AW for ST or serotype and penicillin susceptibility was, respectively, 0.785 (CI95%: 0.729–0.841) and 0.389 (CI95%: 0.326–0.452), while the AW for ST or serotype and erythromycin susceptibility was, respectively, 0.711 (CI95%: 0.598–0.824) and 0.315 (CI95%:0.217–0.413). The sequence types that were associated with penicillin non-susceptible pneumococci (PNSP) and erythromycin resistant pneumococci (ERP) are presented in Table III.4.

Table III.4 Sequence types that were positively associated with penicillin non-susceptibility, erythromycin resistance and erythromycin and penicillin non-susceptibility simultaneously.

Antimicrobial resistance[a]	ST	Yes	No	OR[b] (95% CI)	p-value[c]	Penicillin MIC range (µg/ml)
PNSP	156	34	1	153.83 (25.29-6036.47)	<0.001	0.5-3
	276	31	0	Inf (34.58-Inf)	<0.001	0.19-3
	143	12	0	Inf (10.84-Inf)	<0.001	0.75-3
	338	10	0	Inf (8.65-Inf)	<0.001	0.064-0.19
	63	8	0	Inf (6.52-Inf)	<0.001	0.094-1
	386	7	0	Inf (5.48-Inf)	<0.001	0.064-0.19
	179	7	4	6.69 (1.68-31.50)	<0.001	0.047-2
	230	6	0	Inf (4.45-Inf)	<0.001	0.38-0.75
	2944	5	0	Inf (3.45-Inf)	<0.001	2-8
ERP	276	30	1	150.50 (24.56-5946.55)	<0.001	0.19-3
	179	11	0	Inf (10.96-Inf)	<0.001	0.047-2
	143	10	2	21.90 (4.60-206.95)	<0.001	0.75-3
	717	9	0	Inf (8.52-Inf)	<0.001	0.008-0.032
	9	8	0	Inf (7.32-Inf)	<0.001	0.016-0.064
	63	8	0	Inf (7.32-Inf)	<0.001	0.094-1
	386	7	0	Inf (6.15-Inf)	<0.001	0.064-0.19
	350	5	0	Inf (3.87-Inf)	<0.001	0.004-0.023
	230	5	1	21.27 (2.36-1006.44)	0.001	0.38-0.75
EPNSP	276	30	1	274.20 (44.39-10466.80)	<0.001	0.19-3
	143	10	2	36.81 (7.69-350.14)	<0.001	0.75-3
	63	8	0	Inf (12.17-Inf)	<0.001	0.064-0.19
	386	7	0	Inf (10.19-Inf)	<0.001	0.064-0.19
	179	7	4	12.52 (3.12-59.31)	<0.001	0.047-2
	230	5	1	35.15 (3.88-1669.53)	<0.001	0.38-0.75
	4575	3	0	Inf (2.83-Inf)	0.002	2-3

[a]PNSP—Penicillin non-susceptible pneumococci, ERP—Erythromycin resistant pneumococci, EPNSP—Erythromycin and penicillin non-susceptible pneumococci. [b]OR—odds ratio. INF—infinite. [c]Only significant values after FDR correction are shown.

Discussion

In spite of several years of PCV7 use in children, the most frequent CC was CC156 (11.6%, Table III.1), a lineage that expressed mainly PCV7 serotypes (89.1%) and which was also the most frequent in IPD in the pre-PCV7 period (Serrano et al., 2005). We had previously shown that the serotype distribution of pneumococci causing adult IPD had changed significantly in the post-PCV7 period, with the proportion of PCV7 serotypes declining to values below 20% (Aguiar et al., 2008a; Horácio et al., 2012; Horácio et al., 2013). Adult vaccination with anti-pneumococcal vaccines was low to negligible and prior work indicated that these changes were due to a combination of secular trends and herd effect from children vaccination, which although occurring through the private market reached a coverage of 75% of children ≤ 2 yrs in 2008 (Aguiar et al., 2008a; Horácio et al., 2012; Horácio et al., 2013). Due to these changes one could expect that CC156 would also decrease (this CC accounted for 21.7% of all IPD in 1999–2003 (Serrano et al., 2005)) and potentially lose its dominance. During the study period CC156 accounted for an approximately constant proportion of the characterized isolates in each year (varying slightly between 5.5% and 7.0%). The observed persistence of this CC may be explained by three different factors: 1) while it is true that PCV7 serotypes have declined in importance, it is also true that they still account for approximately one fifth of adult IPD and 57% of the isolates expressing PCV7 serotypes in 2008–2011 belonged to this CC; 2) this CC is strongly associated with antimicrobial resistance, with n = 70/101 isolates being resistant to at least two different classes of antibiotics; and 3) the genomic diversity of CC156 is high, with one study reporting the presence of 10 unrelated genetic subgroups (Moschioni et al., 2013), suggesting that this CC may be particularly suited to adapt to different selective pressures. Regarding the last point, in our study we found representatives of three different clones recognized by the PMEN included in CC156: Spain9V-156, Colombia23F-338 and Greece6B-273 (McGee et al., 2001).

Overall, the clones recognized by the PMEN were strongly represented in our collection with up to 67.3% of the isolates being at most DLVs of one of the 29 different PMEN clones identified. Among the 22 major CCs occurring in the study period (Table III.1), only six did not include a PMEN clone: CC433, CC378, CC460,

CC260, CC30 and CC404. The most frequent of these, CC433 (mainly ST433, Table III.1), was the eighth most frequent CC, included mostly isolates susceptible to antimicrobials, and is now an important cause of IPD worldwide (Ardanuy et al., 2009; Pichon et al., 2013; Golden et al., 2015; Metcalf et al., 2016; Nakano et al., 2016).

The eight more frequent CCs (Table III.1) were mainly composed of isolates expressing one of the top 10 serotypes causing adult IPD in 2008–2011, excluding serotype 4 that presented a high genetic diversity and no dominant CC (Fig. III.1). In fact, the clonal composition of the 10 most frequent serotypes causing adult IPD in Portugal in 2008–2011 (Figs III.1 and III.2) presented both similarities and differences with other geographic regions in similar periods, with most matching results coming from countries in Europe and the Americas, especially for serotypes 3 (Netherlands3-180), 7F (Netherlands7F-191), 22F (ST433) and 9N (ST66) (Ardanuy et al., 2009; Muñoz-Almagro et al., 2011; Yildirim et al., 2012; Pichon et al., 2013; Caierão et al., 2014; Golden et al., 2015; Metcalf et al., 2016). Most of these lineages, with the exception of ST66, were also dominant among isolates expressing the same serotypes and causing IPD in children in Japan (Nakano et al., 2016). Among the isolates expressing serotypes 19F and 23F, the lineages that dominated in the present study where either absent or represented a minority of the isolates of the same serotype in the recent studies from the United States and Japan (Metcalf et al., 2016; Nakano et al., 2016), indicating the persistence of different lineages expressing PCV7 serotypes in different countries. Serotype 19A, which increased as a cause of IPD after PCV7 implementation in several countries, was associated in Portugal with the expansion of the PMEN clone Denmark14-230 while in the USA and Asia it was associated with the emergence of the PMEN clone Taiwan19F-236, as previously described (Aguiar et al., 2010b). Serotype 1 was mostly represented by the Sweden1-306 European clone (Brueggemann and Spratt, 2003). However, we detected for the first time in Portugal two serotype 1 isolates belonging to the hypervirulent PMEN clone Sweden1-217 (STs 217 and 3081), which has been responsible for epidemics with high mortality in Africa (Brueggemann and Spratt, 2003; Harvey et al., 2011). The detection of these genotypes in Portugal is not surprising, since they were found in neighboring Spain (Muñoz-Almagro et al., 2011) and Portugal has a significant community of citizens of African descent. Still, the two isolates detected were collected in 2011, the last year of the study

period, so it will be important to monitor the potential emergence of this genotype as a cause of adult IPD in Portugal. Serotypes 14 and 8 were found mainly among representatives of Spain9V-156 and Netherlands8-53, respectively, similarly to Spain (Muñoz-Almagro et al., 2011). Serotype 11A was found mainly among representatives of ST408 in our study, while the most common lineage in both Spain and the USA was its SLV, ST62 (Muñoz-Almagro et al., 2011; Metcalf et al., 2016). For serotype 4, in spite of the higher diversity some similarity was also found with Spain, with Sweden4-205 and ST246 being common to the two collections of isolates (Muñoz-Almagro et al., 2011).

When comparing our results with those from a recent carriage study in adults in Portugal (Almeida et al., 2014) in addition to the difference in serotype distribution due to the recognized differences in invasiveness of the various serotypes (Sá-Leão et al., 2011), there was also a marked difference between the clonal compositions of serotype 19A, since the majority of isolates expressing this serotype among asymptomatic carriers represented ST1201 (CC15), while in our study the most frequent was ST276, indicating possible differences in virulence between these two serotype 19A lineages.

After the introduction of PCV7, several studies documented a general decrease in IPD incidence. However, the benefits of vaccination were also partly overcome by increases in incidence of non-vaccine serotypes (Aguiar et al., 2010a; Pilishvili et al., 2010; Aguiar et al., 2014; Waight et al., 2015). This could occur through the persistence of a successful lineage now expressing a different serotype not covered by the conjugate vaccines, a phenomenon described as capsular switching. Among our collection a notable case of possible capsular switching was the detection of five isolates related to the PMEN clone Denmark14-230 (ST230, n = 4 and ST4253, n = 1) expressing the non-PCV13 serotype 24F (Table III.2). This combination has already been reported in Portugal in colonized children (Simões et al., 2011), in Italy (Pantosti et al., 2002), Spain and other European countries (http://pubmlst.org/). In Portugal, in the pre-PCV7 period, serotype 24F was predominantly CC81 and mostly susceptible to antimicrobials. In 2008–2011, among the nine isolates genotyped, four represented CC81 and were mainly antimicrobial susceptible as before, while five represented CC230 and were EPNSP. The detection of this genotype expressing serotype 24F in

Portugal is of concern since ST276, an SLV of ST230, was behind the expansion of serotype 19A as a cause of IPD in Portugal in the post-PCV7 era (Aguiar et al., 2010b). Among other possible capsular switches detected in our collection (Table III.2), most reflected the occasional detection of a single isolate of a different serotype, suggesting that even if these result from capsular switching they did not persist in the population at a significant frequency. Taken together this data indicates that capsular switching in our collection was infrequent and cannot be attributed to vaccine pressure, in agreement with other studies (Ramirez and Tomasz, 1999; Wyres et al., 2013). However, even though these events were rare they can be important since the uncommon combinations may proliferate in the future if the conditions become favorable maintaining successful clones in circulation.

Clonal expansion of previously less frequent lineages was a major contributor to the expansion of non-PCV7 serotypes, since the 22 most frequent CCs occurring in 2008–2011 (Table III.1) were already in circulation in 1999–2003 (Serrano et al., 2005). When comparing these two periods the most relevant changes were the expansion of CC191 (serotype 7F) and CC439 (serotypes 23B and 23A) and the decline of CC260 and CC458 (both associated with serotype 3), CC1381 (serotype 18C) and that of CC156 discussed above. The variations in frequency of CC191, CC439 and CC1381 followed the changes occurring in the respective serotypes. Regarding the clonal composition of serotype 3, we found that the decrease in CC260 and CC458 was accompanied by an expansion of CC180 among serotype 3 isolates, explaining the relative stability of this serotype among IPD in adults (Horácio et al., 2013), with CC180 accounting for 40% of serotype 3 IPD in 1999–2003 but for 64% in 2008–2011. Given that isolates belonging to CC180, CC260 or CC458 were mostly susceptible to all tested antimicrobials and that only one isolate from CC180 and another from CC458 carried a PI, this different behavior in time cannot be attributed to differences in these characteristics.

The presence and type of the PIs was more strongly associated with genotype than with serotype, as previously reported (Aguiar et al., 2008b). The genotypes that carried PIs in our study (Table III.3) were essentially the same reported recently in USA (Metcalf et al., 2016), although the proportions of these genotypes differed considerably between the two studies. The proportion of PI-1 carrying isolates increased in the post-PCV7 period in the USA associated with the emergence of the

non-PCV7 serotypes 19A and 35B (Regev-Yochay et al., 2010). Although serotype 19A also increased in Portugal, the genotype behind this increase does not carry a PI (ST276) and an actual decrease of PI-1 positive isolates occurred when compared to the pre-PCV7 period, when 24% of the adult isolates presented PI-1 (Aguiar et al., 2008b). The proportion of isolates presenting only PI-2 declined during the study period, from 25% in 2008 to 15% in 2011. This was expected since serotype 1 isolates are significantly associated with PI-2 and these decreased as a cause of adult IPD during the study period (Horácio et al., 2013). Since PCV13 also includes serotype 7F, which in Portugal was strongly associated with PI-2, continued use of PCV13 may further reduce the proportion of isolates carrying PI-2. In 2011, the proportion of isolates carrying any of the PIs was down to 26.6% of the isolates. As suggested for isolates causing IPD in children (Aguiar et al., 2012), continued PCV13 use has the potential to virtually eliminate PI carrying isolates.

Antimicrobial resistance is not a crucial pre-requisite for the success of serotypes in IPD, as demonstrated by serotypes 1, 3 and 7F that were frequent in the post-PCV7 period and are mostly susceptible to antimicrobials. Still, the presence of resistant clones may help the persistence of serotypes targeted by vaccines, as was possibly the case with serotypes 14 and 19A. The highest proportions of penicillin and erythromycin resistance among adult IPD since the beginning of epidemiological surveillance were registered in 2010, although these declined again in 2011 (Horácio et al., 2013). Between 2008 and 2009, when only the increase in PNSP was significant, this was due to an increase in PNSP expressing serotypes 14 and 19A. In contrast, between 2009 and 2010, the increase in both PNSP and ERP was due to an increase in genetically unrelated resistant isolates expressing different serotypes. Since the number of isolates collected yearly between 2008 and 2011 did not suffer significant fluctuations, two possibilities could explain the initial increase in PNSP isolates expressing serotypes 14 and 19A: 1) an increase in the overall proportions of serotypes 14 and 19A, including PNSP STs or 2) an increase in the proportion of PNSP STs within each of these serotypes, with a concomitant decrease of susceptible STs. Regarding serotype 14 isolates, which increased slightly during the study period, these were by 2008 almost equally distributed into only two CCs: CC15, which includes ST409 and that is almost entirely penicillin susceptible, and CC156, in which all serotype 14

isolates were PNSP. From 2009 onwards, CC156 became the dominant lineage, accounting for over 90% of the isolates expressing serotype 14, a change that was not only due to a decline in frequency of CC15 but also to a slight overall increase in frequency of CC156 among all adult IPD isolates. Among serotype 19A isolates, the increase in proportion of PNSP between 2008 and 2009 was due to the disappearance of ST193, which was fully susceptible to penicillin, and to an increase of ST276, which represented solely PNSP isolates (Table III.4). Although PNSP and ERP returned in 2011 to values similar to those found prior to 2010, this was due to a decrease in frequency of resistant isolates representing multiple STs and expressing different serotypes, while the emerging clones (CC156 among serotype 14 and ST276 among serotype 19A) persisted as important causes of adult IPD. Continued surveillance of resistant isolates should focus particularly on the evolution of serotype 24F since ≥ 50% of the isolates expressing this serotype in our study were associated with the PMEN clone Denmark14-230 (III.S2 Table) which was a major clone in the expansion of serotype 19A in the post-PCV7 period in Portugal.

The significant differences in genetic variation, as documented here by MLST, within the various serotypes remain unexplained and should be the object of future study. We have shown that the changes in serotypes occurring during the study period have been driven mostly by the expansion of previously circulating clones or to declines in the majority of the lineages expressing a given serotype. However, in some serotypes, such as 14 and 19A, changes in serotype frequency were driven mostly by changes in particular lineages. In the case of serotype 3, although its proportion remained constant with time, there were significant changes in the dominant lineages. These observations raise the possibility that lineage-specific properties may condition the dynamics of particular serotypes. Serotype switching played a minor role in this population but may be an important source of new variants that may increase in the post PCVs period. Taken together, these observations reinforce the importance of determining the clonal lineages of pneumococci to better understand the changes in the bacterial population occurring following the use of PCVs.

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Author Contributions

Conceived and designed the experiments: JMC MR. Performed the experiments: ANH CSC JDM JPL. Analyzed the data: ANH JMC MR. Contributed reagents/materials/analysis tools: PGSSI. Wrote the paper: ANH CSC JMC MR.

Supporting Information

Table III.S1 Age distribution and serotypes of the STs found in CCs with less than 10 isolates.

CC (no.isolates)	ST (no. Isolates)	no. of isolates per age group			Serotypes (no.isolates)
		[18-49]	[50-64]	>=65	
717 (9)	717 (9)	0	3	6	33A (7), 33F (1), 3 (1)
994/6158 (9)	994 (7)	1	1	5	19A (7)
	4197 (2)	0	0	2	19A (2)
235 (8)	235 (5)	2	1	2	20 (5)
	1483 (1)	0	1	0	20 (1)
	7221 (1)	0	0	1	20 (1)
	10047 (1)	0	0	1	20 (1)
395 (7)	395 (5)	2	1	2	6C (5)
	1692 (2)	0	0	2	6C (2)
205 (6)	205 (6)	4	0	2	4 (6)
5902 (6)	1222 (4)	4	0	0	4 (4)
	801 (2)	2	0	0	4 (2)
	5902 (1)	1	0	0	16F (1)
241 (5)	241 (5)	2	0	3	18A (4), 19A (1)
473 (5)	1876 (2)	0	1	1	6A (2)
	473 (1)	0	0	1	6B (1)
	1135 (1)	0	0	1	23B (1)
	10055 (1)	1	0	0	6A (1)
1221 (5)	1221 (5)	2	2	1	4 (5)
393 (4)	393 (4)	0	0	4	25A (4)
989 (4)	989 (4)	1	2	1	12B (4)
1816 (4)	1342 (3)	1	0	2	29 (3)
	2567 (1)	0	0	1	29 (1)
320 (3)	320 (2)	0	1	1	19A (2)
	271 (1)	0	0	1	19F (1)
1026 (3)	1026 (3)	1	1	1	20 (3)
1046 (3)	1046 (2)	1	0	1	34 (2)
	8967 (1)	0	0	1	34 (1)
1652/6956 (3)	1652 (3)	0	3	0	4 (3)
2669 (3)	2669 (2)	0	1	1	19A (2)
	2102 (1)	0	0	1	19A (1)
2690 (3)	2690 (3)	0	0	3	35B (3)
198 (2)	198 (1)	0	0	1	35B (1)
	3329 (1)	1	0	0	35B (1)
217 (2)	217 (1)	1	0	0	1 (1)
	3081 (1)	0	1	0	1 (1)
242 (2)	242 (2)	0	0	2	19A (2)
452 (2)	452 (1)	0	1	0	35B (1)
	9979 (1)	0	1	0	29 (1)
546 (2)	494 (2)	0	1	1	28A (2)
1381 (2)	1233 (2)	2	0	0	1 (1), 18C (1)
2021 (2)	1116 (1)	1	0	0	3 (1)
	1126 (1)	0	1	0	39 (1)
2599 (2)	2599 (2)	1	0	1	17A (2)
9958 (2)	9958 (2)	1	0	1	18A (1), 20 (1)

Table III.S1 (continued).

CC (no.isolates)	ST (no.isolates)	no. of isolates per age group			Serotypes (no.isolates)
		[18-49]	[50-64]	>=65	
1368 (2)	1368 (2)	0	1	1	35F (2)
100 (2)	100 (2)	0	0	2	33A (1), 33F (1)
70 (1)	70 (1)	1	0	0	13 (1)
87 (1)	89 (1)	0	1	0	19F (1)
102 (1)	102 (1)	1	0	0	18C (1)
259 (1)	259 (1)	0	1	0	4 (1)
343 (1)	10049 (1)	0	0	1	15A (1)
432 (1)	432 (1)	1	0	0	21 (1)
458 (1)	458 (1)	0	1	0	3 (1)
558 (1)	558 (1)	0	1	0	35B (1)
901 (1)	901 (1)	0	1	0	13 (1)
1010 (1)	9955 (1)	0	0	1	11A (1)
1025 (1)	1025 (1)	0	0	1	15B/C (1)
1151 (1)	2732 (1)	0	0	1	19A (1)
1390 (1)	1390 (1)	0	0	1	6C (1)
1866 (1)	1866 (1)	0	0	1	4 (1)
1884 (1)	478 (1)	1	0	0	NT (1)
2658 (1)	2658 (1)	0	0	1	13 (1)
3214 (1)	3214 (1)	0	0	1	35F (1)
3982 (1)	3982 (1)	0	0	1	9N (1)
6182 (1)	6182 (1)	0	0	1	36 (1)
7069 (1)	7069 (1)	0	0	1	15A (1)
8153 (1)	5823 (1)	0	1	0	28F (1)
9957 (1)	9957 (1)	0	1	0	6B (1)
9970 (1)	9970 (1)	0	1	0	6B (1)
10043 (1)	10043 (1)	1	0	0	29 (1)
10051 (1)	10051 (1)	0	0	1	6B (1)
1083/7843 (1)	1083 (1)	1	0	0	25F (1)
1715/1640 (1)	1715 (1)	1	0	0	6C (1)
6973/2668 (1)	6973 (1)	0	1	0	19A (1)

Table III.S2 Distribution of STs according to serotype of the isolates (n≤11) causing adult IPD in 2008-2011 and expressing serotypes not included in any of the conjugate vaccines.

Serotype (no. isolates)	ST (no. isolates)
15A (11)	63 (7)
	1228 (1)
	3130 (1)
	7069 (1)
	10049 (1)
15B/C (11)	199 (2)
	411 (7)
	1025 (1)
	3863 (1)
23B (11)	439 (7)
	1135 (1)
	9579 (1)
	10039 (2)
24F (9)	72 (4)
	230 (4)
	4253 (1)
29 (8)	198 (1)
	558 (1)
	1342 (3)
	2567 (1)
	9979 (1)
	10043 (1)
33A (8)	100 (1)
	717 (7)
17F (7)	123 (5)
	6179 (1)
	9966 (1)
31 (6)	1766 (5)
	1994 (1)
35F (6)	1366 (2)
	1368 (2)
	3214 (1)
	4849 (1)
Non-typable (6)	53 (2)
	66 (1)
	191 (2)
	478 (1)
	1046 (2)
	2001 (2)
	8967 (1)
34 (5)	1046 (2)
	2001 (2)
	8967 (1)

Table III.S2 (continued).

Serotype (no. Isolates)	ST (no. Isolates)
35B (5)	452 (1) 2690 (3) 3329 (1)
18A (5)	241 (4) 9958 (1)
18C (5)	102 (1) 113 (1) 199 (1) 1233 (1) 10033 (1)
7C (4)	1201 (3) 9956 (1)
25A (4)	393 (4)
21 (4)	432 (1) 1877 (3)
17A (3)	392 (1) 2599 (2)
13 (3)	70 (1) 901 (1) 2658 (1)
28A (2)	494 (2)
33F (2)	100 (1) 717 (1)
15F (1)	63 (1)
36 (1)	6182 (1)
11C (1)	408 (1)
25F (1)	1083 (1)
24A (1)	162 (1)
28F (1)	5823 (1)
39 (1)	1126 (1)
12F (1)	220 (1)
7A (1)	191 (1)

CHAPTER IV: NON-INVASIVE PNEUMOCOCCAL PNEUMONIA – SEROTYPES AND ANTIMICROBIAL SUSCEPTIBILITY

RATIONALE

In the studies presented in previous chapters we analyzed pneumococci responsible for invasive disease in adults. Now, in the two studies included in the present chapter (Horácio et al., 2014; Horácio et al., 2018) we focused our attentions on pneumococci causing non-invasive pneumonia in adults. The importance of NIPP is unquestionable. NIPP is the most common cause of hospitalization and death due to a pneumococcal disease in adults and this is largely due to the high incidence and relatively high severity of this disease. There were no previous publications from the laboratory evaluating isolates causing adult NIPP in Portugal and therefore the studies presented in this chapter evaluate adult NIPP isolates recovered since 1999. The first study includes a random sample of 100 isolates per year from a collection of adult NIPP isolates recovered from 1999 to 2011 (Horácio et al., 2014), while the second study includes all adult NIPP isolates collected from 2012 to 2015 (Horácio et al., 2018).

The following study was performed by Andreia N. Horácio, Joana P. Lopes, Mário Ramirez, José Melo-Cristino and the Portuguese Group for the Study of Streptococcal Infections. Andreia N. Horácio performed a significant part of the experimental work, most of the statistical analysis and wrote the first version of the manuscript.

NON-INVASIVE PNEUMOCOCCAL PNEUMONIA IN PORTUGAL — SEROTYPE DISTRIBUTION AND ANTIMICROBIAL RESISTANCE[1]

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Abstract

There is limited information on the serotypes causing non-invasive pneumococcal pneumonia (NIPP). Our aim was to characterize pneumococci causing NIPP in adults to determine recent changes in serotype prevalence, the potential coverage of pneumococcal vaccines and changes in antimicrobial resistance. Serotypes and antimicrobial susceptibility profiles of a sample of 1300 isolates recovered from adult patients (≥ 18 yrs) between 1999 and 2011 (13 years) were determined. Serotype 3 was the most frequent cause of NIPP accounting for 18% of the isolates. The other most common serotypes were 11A (7%), 19F (7%), 19A (5%), 14 (4%), 22F (4%), 23F (4%) and 9N (4%). Between 1999 and 2011, there were significant changes in the proportion of isolates expressing vaccine serotypes, with a steady decline of the serotypes included in the 7-valent conjugate vaccine from 31% (1999–2003) to 11% (2011) ($p < 0.001$). Taking together the most recent study years (2009–2011), the potential coverage of the 13-valent conjugate vaccine was 44% and of the 23-valent polysaccharide vaccine was 66%. While erythromycin resistance increased from 8% in 1999–2003 to 18% in 2011 ($p < 0.001$), no significant trend was identified for penicillin non-susceptibility, which had an average value of 18.5%. The serotype distribution found in this study for NIPP was very different from the one previously described for IPD, with only two serotypes in common to the ones responsible for half of each presentation in 2009–2011 – serotypes 3 and 19A. In spite of these differences, the overall prevalence of resistant isolates was similar in NIPP and in IPD.

[1] A *facsimile* of this publication can be found at the "Publications" section of this thesis.

Introduction

Pneumonia is a common infection that causes high rates of morbidity and mortality worldwide. *Streptococcus pneumoniae* (pneumococcus) is thought to be the major cause of pneumonia, responsible for up to half of all cases (Polverino et al., 2013). Only a small fraction of pneumococcal pneumonias are bacteremic, with non-invasive pneumococcal pneumonia (NIPP) estimated to be three to ten times more frequent than invasive pneumococcal pneumonia (Said et al., 2013; Rodrigo et al., 2014). While bacteremic pneumonia is a more severe form of pneumonia, it is less clear if bacteremia can be considered an independent predictor of mortality (Cillóniz and Torres, 2012; Benfield et al., 2013). In adults, bacteremic pneumonia accounts for most of the cases of invasive pneumococcal disease (IPD). While the serotype distribution of IPD and NIPP have been sometimes assumed to be the same (Smith et al., 2012), it is becoming increasingly clear that this is not so (Benfield et al., 2013; Sherwin et al., 2013). This observation is in agreement with the recognition that some serotypes, and even different genetic lineages expressing the same serotype, may have different invasive disease potentials (Sá-Leão et al., 2011), leading to the expectation that less invasive serotypes would be more abundantly represented in NIPP than in IPD.

In developed countries, pneumonia is believed to be a major cause of morbidity among older adults, and, together with influenza, is the leading cause of death from infectious disease in the US considering the entire population (Heron, 2013). Until recently, the only available vaccine for adults was the 23-valent polysaccharide vaccine (PPV23) that, while potentially effective in preventing IPD, may be less efficacious against NIPP (Grabenstein, 2012; Trück et al., 2012). Possibly due to the ongoing debate on the usefulness of PPV23 vaccination, in the majority of the European countries, including Portugal, there has been a low uptake of this vaccine (Fedson et al., 2011; Horácio et al., 2012). On the other hand, the 7-valent conjugate vaccine (PCV7) was introduced in many European countries for vaccinating children, rapidly reaching high coverages in the targeted age groups. PCV7 was available in Portugal for vaccination of children between 2001 and 2009 and, although not part of the national immunization program, its uptake was estimated to have grown continuously, albeit slower that in countries where it was part of the national

immunization program (Aguiar et al., 2008a). Changes in the serotype distribution of isolates causing IPD, compatible with an effect of PCV7 occurred in both children and adults in Portugal and elsewhere (Aguiar et al., 2008a; Aguiar et al., 2010a; Horácio et al., 2012; Steens et al., 2013; Regev-Yochay et al., 2014), the latter potentially resulting from a herd effect. The 10-valent (PCV10) and the 13-valent conjugate vaccines (PCV13) became available in Portugal in 2009 and in 2010, respectively. In September 2011, PCV13 received the European Medicines Agency approval for use in adults ≥ 50 yrs and in July 2013 was approved for adults ≥ 18 yrs, for the prevention of IPD. Currently PCV13 is approved for all ages from 6 weeks up and there is now initial evidence of its efficacy against pneumococcal pneumonia in adults caused by the serotypes included in the vaccine (Bonten et al., 2014).

The aim of this study was to determine the serotype distribution and antimicrobial resistance of pneumococci causing NIPP in adults in Portugal during a 13-year period, when the three conjugate vaccines were available for children, and to compare these data to the information available for IPD published previously (Horácio et al., 2013).

Materials and Methods

Ethics Statement

Case reporting and isolate collection were considered to be surveillance activities and were exempt from evaluation by the Review Board of the Faculdade de Medicina da Universidade de Lisboa. The data and isolates were de-identified so that these were irretrievably unlinked to an identifiable person.

Bacterial Isolates

Isolates were provided by a laboratory-based surveillance system that includes 30 microbiology laboratories throughout Portugal. These were asked to identify and send to our laboratory all pneumococci causing infections. Although the laboratories were contacted periodically to submit the isolates to the central laboratory, no audit was performed to ensure compliance, which may be variable in this type of study. After arrival, all isolates were confirmed as *S. pneumoniae* by colony morphology and hemolysis on blood agar plates, optochin susceptibility and bile solubility.

The isolates included in this study were recovered from adult patients (≥ 18 yrs) with a clinical diagnosis of pneumonia between 1999 and 2011. A total of 1300 isolates, 100 isolates randomly chosen from among the isolates received in each of the 13 years of the study were included. The total number of isolates submitted to the central laboratory in each year was 161 in 1999, 184 in 2000, 319 in 2001, 282 in 2002, 265 in 2003, 341 in 2004, 338 in 2005, 392 in 2006, 525 in 2007, 601 in 2008, 473 in 2009, 519 in 2010 and 445 in 2011. We believe this reflects increasing compliance with the surveillance activities with time. Only isolates recovered from sputum, bronchial secretions or bronchoalveolar lavage were considered. Isolates were not included when pneumococci were simultaneously isolated from blood or another usually sterile product, and when other potential bacterial pathogens besides pneumococci were detected in the sample (such as *Haemophilus influenzae* that was frequently detected). Only one isolate from each patient in each year was considered. Among the 1300 isolates selected, 103 (7.9%) were isolates from bronchoalveolar lavage fluid.

Serotyping and Antimicrobial Susceptibility Testing

Serotyping was performed by the standard capsular reaction test using the chessboard system and specific sera (Statens Serum Institut, Copenhagen, Denmark). Serotypes were grouped into conjugate vaccine serotypes, i.e., those included in PCV13 (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 19A, 23F) that comprise all the serotypes found in the lower valency vaccines (PCV7: 4, 6B, 9V, 14, 18C, 19F, 23F; and PCV10: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F), those included in PPV23 (all serotypes included in PCV13 except 6A and serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F), and non-vaccine serotypes (NVT). The isolates that were not typable with any of the complete set of sera were considered non-typable (NT).

Minimum inhibitory concentrations (MICs) for penicillin and cefotaxime were determined using Etest strips (Biomérieux, Marcy-L'Etoile, France). The results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) recommended breakpoints prior to 2008 (Clinical and Laboratory Standards Institute, 2007), as these allows the comparison with previously published data. According to these criteria, intermediate level penicillin resistance is defined as MIC 0.12–1.0 μg/ml and high level resistance as MIC ≥ 2.0 μg/ml. Isolates that fell into either one of these classes were designated penicillin non-susceptible. Susceptibility to cefotaxime was defined as MIC ≤ 1.0 μg/ml. The Kirby-Bauer disk diffusion assay was used to determine susceptibility to levofloxacin, erythromycin, clindamycin, chloramphenicol, trimethoprim/sulphamethoxazole, tetracycline, vancomycin and linezolid, according to the CLSI recommendations and interpretative criteria (Clinical and Laboratory Standards Institute, 2013). Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin, as previously described (Melo-Cristino et al., 2003). The MLS_B phenotype (resistance to macrolides, lincosamides and streptogramin B) was defined as the simultaneous resistance to erythromycin and clindamycin, while the M phenotype (resistance to macrolides) was defined as non-susceptibility only to erythromycin.

Statistical Analysis

Sample diversity was measured using Simpson's index of diversity (SID) and the respective 95% confidence intervals (CI95%) (Carriço et al., 2006). To compare two

sets of partitions the Adjusted Wallace (AW) coefficients were calculated (Severiano et al., 2011) using the online tool available at www.comparingpartitions.info. Differences were evaluated by the Fisher exact test with the false discovery rate (FDR) correction for multiple testing (Benjamini and Hochberg, 1995) or the Chi-squared test, and the Cochran-Armitage test was used for trends. A $p < 0.05$ was considered significant for all tests.

Results

Serotype Distribution

Serotype diversity was high [SID: 0.941, CI95%: 0.935–0.948], with 57 different serotypes detected among the 1300 isolates. The most frequent serotypes, which accounted for more than half of the isolates, were serotypes: 3 (17.8%), 11A (6.7%), 19F (6.7%), 19A (5.2%), 14 (4.1%), 22F (4.1%), 23F (3.8%) and 9N (3.5%). Serotype distribution in each of the studied years is represented in Figure IVa.1. We chose to represent an average of the yearly values between 1999 and 2003, because it was shown previously that this period corresponded to the years before an effect of children vaccination with PCV7 was noted in the distribution of adult IPD serotypes (Aguiar et al., 2008a). The yearly distribution of the 10 overall most frequent serotypes between 1999 and 2003 is represented in Figure IVa.S1. In spite of yearly variations, serotype diversity was high in all studied years with the lowest SID detected in 2008 [SID: 0.901, CI95%: 0.857–0.945] and the highest value found in both 2004 and 2009 [SID: 0.957, CI95%: 0.840–0.973].

The change in distribution of vaccine types along the study years is shown in Figure IVa.2 and Figure IVa.S2. The serotypes included in PCV7 declined gradually from 31% in 1999–2003 to 11% in 2011 (Cochran-Armitage test of trend $p < 0.001$). Among the PCV7 serotypes, those that contributed mostly to this decline were serotypes 6B (from 4.6% to 0%, Cochran-Armitage test of trend $p < 0.001$), 9V (from 3.2% to 0%, Cochran-Armitage test of trend $p < 0.001$) and 23F (from 6.6% to 1.0%, Cochran-Armitage test of trend $p < 0.001$). Despite fluctuations throughout the study period in the number of isolates representing PCV13 and PPV23 serotypes, no consistent trend was noted. However, a decline of the isolates expressing serotypes included in these vaccines, and a consequent increase in the prevalence of NVTs, can be distinguished between 2008 and 2009. This can be attributed to a fall in serotype 3, from 28% to 9%, between these two years ($p < 0.001$, Figure IVa.1). Although in subsequent years the proportion of isolates expressing serotype 3 returned to values similar to those found previously (Figure IVa.1), this did was not reflected in an increase in the proportion of isolates expressing PCV13 serotypes, which remained close to 43% (Figure IVa.2). In contrast, the decline in the proportion of isolates expressing PPV23 serotypes noted in 2009 was not sustained, with the increases in

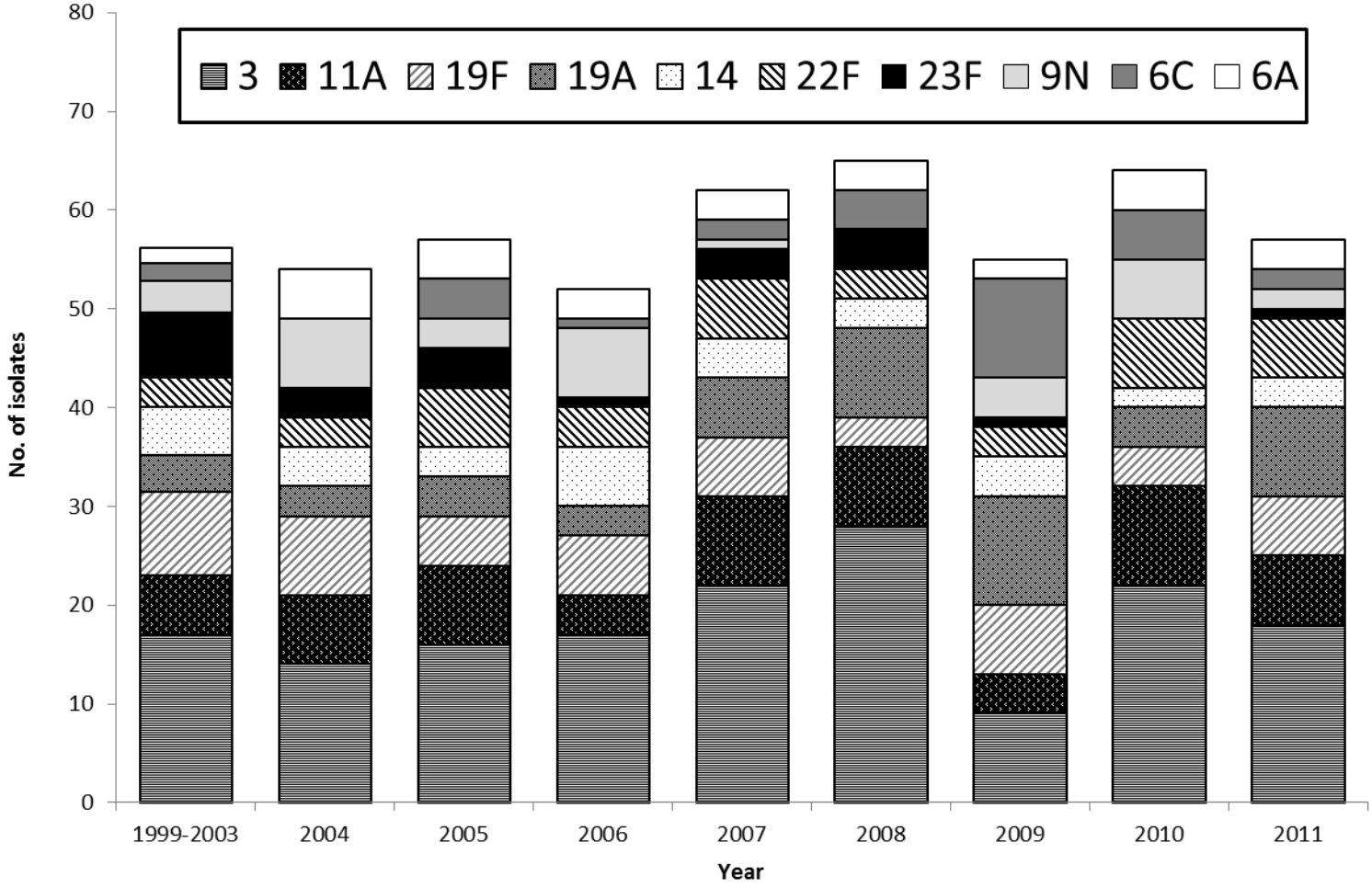

Figure IVa.1 Serotype distribution of the isolates causing non-invasive pneumococcal pneumonia in adults in Portugal (1999–2011). Only the overall 10 most frequent serotypes are shown. The other serotypes found between 1999 and 2011 were serotypes 6B, 7F and 15A (n = 32, each), 23B (n = 30), 15B (n = 28), 10A (n = 27), 9V and non-typable (n = 26, each), 23A (n = 24), 1 (n = 22), 8 (n = 20), 16F (n = 19), 29 (n = 18), 4 (n = 17), 31 (n = 16), 34 (n = 15), 18C (n = 14), 17F and 33A (n = 13, each), 21 and 35F (n = 11, each), 15C and 35C (n = 10, each), 20 (n = 9), 12B and 17A (n = 8, each), 13, 25F and 25A/38 (n = 7, each), 7C and 28A (n = 5, each), 5 (n = 4), 11F, 18A and 19C (n = 3, each), 12A, 12F, 35A, 35B, 38 (n = 2, each) and 9L, 10F, 15F, 16A, 19B, 33F, 39 and 42 (n = 1, each). The value shown for 1999–2003 refers to the yearly average of the 500 isolates studied that were isolated in these 5 years. This period was analyzed together since previously published IPD data indicated that these corresponded to a pre-PCV7 serotype distribution.

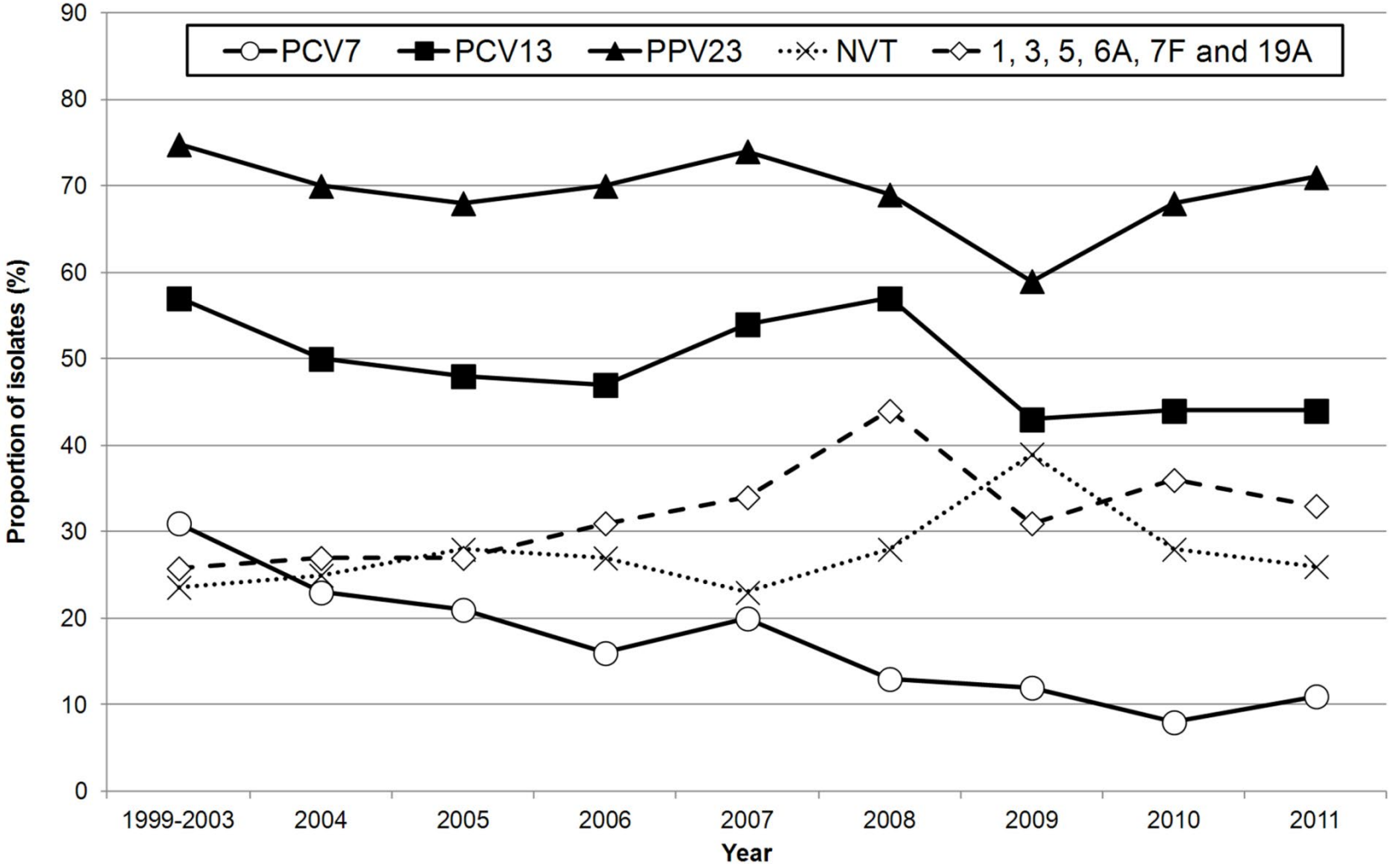

Figure IVa.2 Proportion of isolates expressing serotypes included in pneumococcal vaccines causing non-invasive pneumococcal pneumonia in adults in Portugal (1999–2011). The value shown for 1999–2003 refers to the yearly average of the 500 isolates studied that were isolated in these 5 years. This period was analyzed together since previously published IPD data indicated that these corresponded to a pre-PCV7 serotype distribution.

the following years bringing this value back into the range found in the previous decade (Figure IVa.2).

The distribution of the 10 most frequent serotypes found between 1999 and 2011, stratified by age group, is shown in table IVa.S1. The serotype distribution is similar for each of the age groups considered (p = 0.398) and no significant associations, after correction for FDR, could be found between specific serotypes and age groups. When considering only the three last years of the study, corresponding to the years immediately prior to PCV13 receiving approval for use in adults (2009–2011), the overall proportion of isolates expressing serotypes included in the various vaccines were 10.3% for PCV7, 43.7% for PCV13 and 66.0% for PPV23. There was also no correlation between the proportion of isolates causing NIPP included in the vaccines and the different age groups (table IVa.S2).

Antimicrobial susceptibility

The proportion of isolates resistant to the tested antimicrobials between 1999 and 2011 is summarized in table IVa.1. Penicillin non-susceptible pneumococci (PNSP) accounted for 18.5% of the isolates (n = 241). Among these, 211 isolates (16.2%) expressed low level resistance (MIC = 0.12–1 μg/mL) and 30 isolates (2.3%) high level resistance (MIC ≥ 2 μg/mL). Considering the current CLSI guidelines for parenteral penicillin in non-meningitis cases, where high level is defined as MIC≥8 μg/mL and intermediate resistance as MIC ≥ 2 μg/mL (Clinical and Laboratory Standards Institute, 2013), only 16 strains (1.2%) would have been considered non-susceptible to penicillin, with only one of these expressing high level resistance.

Erythromycin resistant pneumococci (ERP) accounted for 16.3% of the isolates (n = 212), with 84.0% (n = 178) of these expressing the MLS_B phenotype and 16.0% (n = 34) the M phenotype. A total of 9.8% (n = 127) of the isolates were simultaneously non-susceptible to penicillin and resistant to erythromycin (EPNSP).

Resistance to levofloxacin was low overall (1.3%, n = 17), but higher in the older age groups than in the youngest group (Table IVa.1, 18–49 yrs versus 50–64 yrs, p = 0.014 and 18–49 yrs versus ≥ 65 yrs, p = 0.012). No other significant associations with age were found for the other antimicrobials tested. All isolates were susceptible to vancomycin and linezolid. The proportion of erythromycin and clindamycin resistant

isolates increased between 1999–2003 and 2011. Erythromycin resistance increased from 8.0% to 18.0% (Cochran-Armitage test of trend p < 0.001) and clindamycin resistance increased from 7.0% to 15.0% (Cochran-Armitage test of trend p = 0.004). No other significant changes were noted for the other antimicrobials tested.

Table IVa.1 Antimicrobial resistance of the isolates responsible for non-invasive pneumococcal pneumonia in adults in Portugal, stratified by age groups (1999–2011).

	No. resistant isolates (%)[a]			
	[18-49] yrs	[50-64] yrs	≥65 yrs	Total
	(n = 481)	(n = 293)	(n = 526)	(n = 1300)
PEN	95 (19.8)	57 (19.5)	89 (16.9)	241 (18.5)
MIC90	0.25	0.38	0.25	-
MIC50	0.023	0.023	0.023	-
CTX	4 (0.8)	3 (1.0)	2 (0.4)	9 (0.7)
MIC90	0.19	0.125	0.094	-
MIC50	0.012	0.012	0.012	-
LEV	1 (0.2)	6 (2.0)	10 (1.9)	17 (1.3)
ERY	76 (15.8)	54 (18.4)	82 (15.6)	212 (16.3)
CLI	66 (13.7)	44 (15.0)	68 (12.9)	178 (13.7)
CHL	17 (3.5)	12 (4.1)	22 (4.2)	51 (3.9)
SXT	89 (18.9)	46 (15.7)	84 (16.0)	219 (16.8)
TET	61 (12.7)	42 (14.3)	63 (12.0)	166 (12.8)

[a]PEN – penicillin; CTX – cefotaxime; LEV – levofloxacin; ERY – erythromycin; CLI – clindamycin; CHL – chloramphenicol; SXT – trimethoprim/sulphamethoxazole; TET – tetracycline. All isolates were susceptible to vancomycin and linezolid. [b]Non-susceptibitily to penicillin was determined using the CLSI breakpoints prior to 2008.

There was an association between serotype and antimicrobial resistance. The AW for serotype and PNSP was 0.588 (CI95%: 0.541–0.634) and the AW for serotype and ERP was 0.489 (CI95%: 0.419–0.558). Table IVa.2 shows the serotypes that presented at least 10 PNSP and ERP isolates, respectively. Among the major serotypes expressed by PNSP, only serotype 19F was not significantly associated with PNSP. Among the major serotypes expressed by ERP, only serotypes 23F and 6C were not significantly associated with ERP. The PCV7, PCV13 and PPV23 serotypes accounted for 56.0%, 70.5% and 69.7% of PNSP, respectively, and 42.9%, 60.8% and 66.5% of ERP, respectively.

Table IVa.2 Serotype distribution of PNSP and ERP causing non-invasive pneumococcal pneumonia in adults in Portugal (1999–2011).

	Serotype[a]	No. of resistant isolates (%)	OR ($CI_{95\%}$)	p-value[b]
PEN	23F	39 (16.2)	12.6 (6.2-27.7)	**<0.001**
	14	37 (15.4)	8.1 (4.3-15.9)	**<0.001**
	19A	28 (11.6)	2.3 (1.3-3.9)	**0.002**
	15A	27 (11.2)	18.2 (6.8-61.3)	**<0.001**
	19F	27 (11.2)	1.4 (0.8-2.3)	0.193
	9V	23 (9.5)	25.5 (7.6-133.7)	**<0.001**
	6C	18 (7.5)	3.0 (1.5-6.2)	**0.001**
	Others [c]	42 (17.4)	-	-
ERY	19F	37 (17.5)	3.4 (2.1-5.4)	**<0.001**
	19A	31 (14.6)	3.7 (2.2-6.4)	**<0.001**
	15A	28 (13.2)	31.9 (11.0-126.2)	**<0.001**
	14	21 (9.9)	2.8 (1.5-5.1)	**<0.001**
	6B	15 (7.1)	3.7 (1.7-8.0)	**<0.001**
	23F	14 (6.6)	1.6 (0.8-3.1)	0.151
	33A	10 (4.7)	13.8 (3.5-79.0)	**<0.001**
	6C	10 (4.7)	1.5 (0.6-3.3)	0.296
	NT[d]	10 (4.7)	2.6 (1.0-6.1)	**0.025**
	Others[e]	36 (17.0)	-	-

[a]Only the serotypes that presented at least 10 non-susceptible isolates are shown. [b]Significant P-values after FDR correction are highlighted in bold. [c]Other serotypes found among PNSP: 6B (n=8), non-typable (n=7), 6A and 29 (n=5, each), 23B and 24F (n=3, each), 7C (n=2), 1, 3, 4, 11A, 15B, 15F, 22F, 23A, 35A (n=1, each). [d]NT – non typable. [e]Other serotypes found among ERP: 9V and 11A (n=4, each), 3, 15B, 22F, 23A, 24F (n=3, each), 6A (n=2), 1, 7F, 8, 9N, 15F, 16F, 17F, 23B, 29, 33F and 35F (n=1, each).

Discussion

Serotype 3 was the most important serotype in NIPP in adults in Portugal. This serotype was the most frequently detected in all studied years, with the exception of 2009, when it ranked third (Figure IVa.1). A predominance of serotype 3 was also found in two studies that focused on the serotype distribution of pneumococcal pneumonia isolates (Domenech et al., 2011; Benfield et al., 2013), and this serotype was among the most frequent in two recent studies using urinary antigen detection assays to diagnose pneumococcal pneumonia (Huijts et al., 2013; Sherwin et al., 2013), although these last studies included both NIPP and bacteremic pneumonia. In Portugal, serotype 3 is not only a leading cause of NIPP but also of IPD, as can be seen in Figure IVa.S3, showing the distribution of the most frequently detected serotypes immediately prior to the licensure of PCV13 for adult immunization (2009–2011).

Although both IPD and NIPP were characterized by a high serotype diversity (for NIPP SID = 0.943, CI95%: 0.932–0.955; and for IPD SID = 0.942, CI95%: 0.937–0.946; considering 2009–2011), the actual serotype distribution is quite different (Figures IVa.3, Figure IVa.S3 and Table IVa.S3). Among the most frequent serotypes, accounting for half of the characterized isolates in 2009–2011, only two serotypes were common to both NIPP and IPD, which were serotypes 3 and 19A (Figure IVa.S3). When comparing the serotype distribution, serotypes 6A, 11A, 15C, 19F and 23B were significantly more abundant among NIPP isolates, whereas serotypes 1, 4,7F and 14 were significantly more abundant among IPD isolates (Figure IVa.3 and Table IVa.S3). These four serotypes, together with serotype 3, were already shown to have an enhanced invasive disease potential in a study evaluating the serotypes and clones circulating in Portugal (Sá-Leão et al., 2011). On the other hand, serotypes 6A, 11A and 19F were associated with carriage, suggesting their lower invasive disease potential, consistent with the association with NIPP determined here (Figure IVa.3 and Table IVa.S3).

A recent study described the serotype distribution among isolates recovered in 2011 causing bacteremic and non-bacteremic pneumonia in adults in Denmark (Benfield et al., 2013). When considering only the isolates recovered in 2009–2011 in Portugal, the serotype distribution is, perhaps surprisingly, remarkably similar in

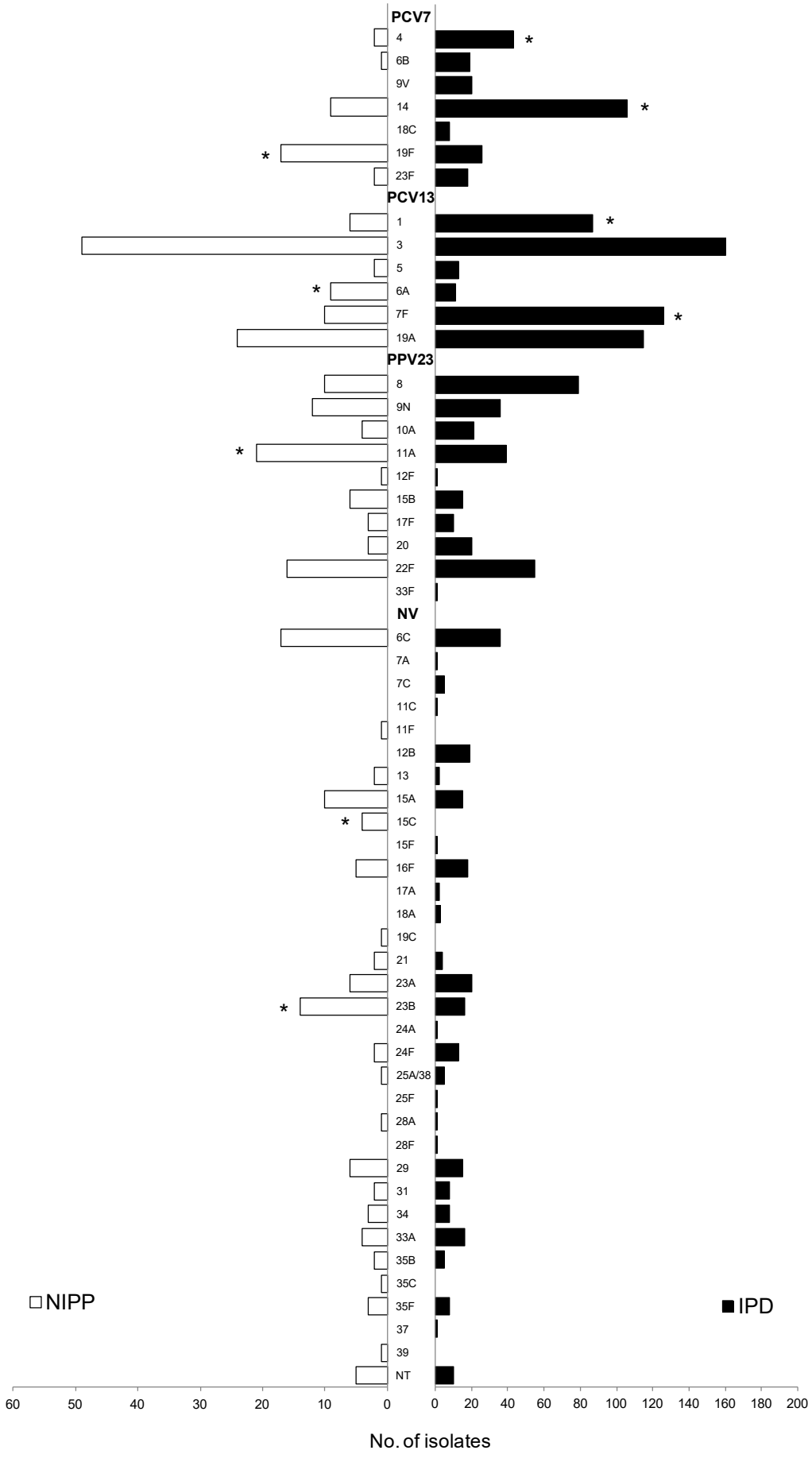
PCV7
4
6B
9V
14
18C
19F
23F
PCV13
1
3
5
6A
7F
19A
PPV23
8
9N
10A
11A
12F
15B
17F
20
22F
33F
NV
6C
7A
7C
11C
11F
12B
13
15A
15C
15F
16F
17A
18A
19C
21
23A
23B
24A
24F
25A/38
25F
28A
28F
29
31
34
33A
35B
35C
35F
37
39
NT
NIPP
IPD
60 50 40 30 20 10 0
0 20 40 60 80 100 120 140 160 180 200
No. of isolates

Figure IVa.3 Serotype distribution of the isolates causing non-invasive pneumococcal pneumonia and invasive pneumococcal disease in adults in Portugal (2009–2011). Data from IPD were published previously (Horácio et al., 2013). Serotypes associated with NIPP or IPD are marked by asterisks. The odds ratio was used to measure the association between serotype and disease presentation and only significant values ($P < 0.05$) after FDR correction are indicated. The P values for the serotypes associated with NIPP were: $P = 0.001$ for 19F, $P = 0.007$ for 6A, $P = 0.004$ for 11A, $P = 0.001$ for 15C, $P < 0.001$ for 23B. The P values for the serotypes associated with IPD were: $P = 0.007$ for 4, $P < 0.001$ for 14, $P < 0.001$ for 1 and $P < 0.001$ for 7F.

both studies. Among the differences are the more significant fractions of non-typable isolates among NIPP and of serotype 1 isolates in bacteremic pneumonia in Denmark relative to Portugal.

Serotype 1 was always an important serotype among isolates causing IPD in both children and adults in Portugal, but its significance has declined in recent years (Aguiar et al., 2010a; Horácio et al., 2012; Horácio et al., 2013; Aguiar et al., 2014). This change cannot be attributed entirely to the use of higher valency vaccines, such as PCV13, although vaccination was potentially a contributing factor (Horácio et al., 2013; Aguiar et al., 2014). Another important difference is the persistence of serotype 14 isolates among both NIPP and IPD in Portugal, in contrast to Denmark where this serotype was found at a lower frequency (Benfield et al., 2013). Serotype 14 was already included in PCV7, and continued vaccine use could be expected to significantly reduce its prevalence. A lower and more protracted vaccine uptake in Portugal compared to Denmark, together with a higher antibiotic consumption, could contribute to the differences observed between the two countries.

In previous studies, we showed that immunization of children with PCV7 resulted in the decline of PCV7 serotypes as causes of adult IPD (Horácio et al., 2012; Horácio et al., 2013). In the present study, we show that a decline of PCV7 serotypes also occurred among isolates causing NIPP in adults. However, while in IPD this decline was abrupt, occurring between 2004 and 2005, in NIPP this decline was gradual and occurred over the entire post-PCV7 period (Figure IVa.2 and Figure IVa.S2). The proportion of isolates expressing PCV7 serotypes in the most recent years of the study was different between NIPP and IPD (10.3% versus 19.0% in 2009–2011, $p < 0.001$, Figure IVa.3).

Taken together, the isolates expressing PCV13 serotypes also declined in IPD and in NIPP, a change that was observed from 2008 onwards for both disease presentations. However, again there were important differences. While in IPD this

decline occurred from 2008 to 2011 and was mostly due to decreases in serotypes 1 and 5, in NIPP this decline occurred between 2008 and 2009 and was predominantly caused by a decrease in serotype 3. In neither case can we attribute these changes to the introduction of PCV13 in Portugal, since this vaccine only became available for children in the beginning of 2010 and received an indication for adults in the beginning of 2012. In NIPP, one possible explanation for the change observed could be the H1N1pdm09 pandemic that occurred between 2009 and 2010. Individuals infected by influenza are at high risk of developing secondary bacterial infections, especially with pneumococci (Smith et al., 2013). Consistent with the hypothesis that influenza allowed the emergence of multiple serotypes as causes of NIPP, the decrease of serotype 3 from 2008 to 2009 was accompanied by an increase in serotype diversity.

Another remarkable difference between NIPP and IPD is the proportion of isolates that expresses serotypes included in the available vaccine formulations with an adult indication. When analyzing data from 2009 to 2011, we found that the number of IPD cases that could have been potentially prevented by PCV13 and PPV23 was 59%, and 80%, respectively (Horácio et al., 2013), while the proportion of isolates expressing these serotypes was only 44% and 66%, respectively, among our collection of NIPP isolates. The higher proportion of vaccine types among IPD isolates was also documented in Denmark (Benfield et al., 2013). The efficacy of the conjugate vaccines is well established and adults could now benefit directly from PCV13 use. However, according to our sample more than half of NIPP cases could not have been prevented by vaccination with PCV13.

In the present study we could not find any significant associations between serotypes and age groups. This is in contrast to our previous studies with invasive isolates, where serotypes 3 and 19A were associated with older patients and serotypes 1 and 8 were associated with younger patients (Horácio et al., 2013). However, if we do not consider the correction for multiple testing, serotype 3 was more frequent in older adults than in the youngest (14% in 18–49 yrs versus 20% in ≥ 50 yrs, $p = 0.005$, Table IVa.S1). The lack of association for the other three serotypes with age is likely the result of their small numbers in our NIPP sample, particularly in what concerns serotypes 1 and 8.

A high proportion of the resistant isolates recovered between 1999 and 2011 are of serotypes included in PCV7 (Table IVa.2), accounting for 56% of PNSP and 43% of ERP in the entire study period. Unlike what could have been expected, the introduction of PCV7 in Portugal did not reduce the proportion of resistant isolates, neither in NIPP nor in IPD (Aguiar et al., 2008a; Horácio et al., 2013). Actually, for both presentations there was an increase in ERP between 1999 and 2011, and for IPD there was also an increase in PNSP. However, when we considered the most recent data (2009 to 2011) we found that only 22% of PNSP and 26% of ERP causing NIPP, represented serotypes included in PCV7. This means that resistant isolates expressing serotypes that are not included in PCV7 have emerged and expanded in the post-PCV7 period.

When considering the entire study period, antimicrobial resistance among NIPP isolates was similar to the values reported recently for IPD (Horácio et al., 2013) (Table IVa.1). Given the association between serotype and antimicrobial resistance, and the different serotype distributions between NIPP and IPD, how can we explain the similar overall resistance? For the most part, the explanation can be found in the more gradual decrease of resistant PCV7 serotypes, albeit to a lower level, in NIPP when compared to IPD. This was accompanied by the rise of a different set of serotypes including resistant isolates that are not included in PCV7, resulting in similar overall resistance levels. Resistance among NIPP isolates is partly due to the proliferation of resistant serotype 19A isolates, probably representing a lineage which has been expanding as a cause of IPD in children and adults (Horácio et al., 2013; Aguiar et al., 2010b), and that became the single most important serotype among PNSP and ERP in the last three years of the study. This was accompanied by increases in serotypes including resistant isolates not represented in PCV13, such as serotypes 6C, 15A, 29, 33A, as well as non-typable isolates, each including $n > 5$ PNSP or ERP during the entire study period (table IVa.2).

The major limitation of this study is that we do not know if blood cultures were performed for all patients, and so we cannot exclude the possibility that some of the isolates attributed to NIPP were in fact reflecting cases of invasive disease. However, the distinct serotype distribution between IPD and NIPP and the similar distribution found in this study and among isolates causing NIPP in Denmark in a similar period

(Benfield et al., 2013), in spite of the different epidemiological contexts, strongly argues against a significant bias in our sample. Another possible confounder could be that a fraction of our isolates are reflecting colonization and not infection. Again we consider this unlikely. The fluids included are not present in healthy subjects (sputum and bronchial secretions) or are not obtained unless there is a strong suspicion of pneumonia (bronchoalveolar lavage). The participating laboratories used criteria to exclude low quality samples, which would be more likely to reflect upper airway microbiota. Finally, adult colonization is known to be rare (Almeida et al., 2014) and would be therefore unlikely to account for a significant fraction of the isolates. Taken together, these arguments support a role for the pneumococci analyzed in infection and not asymptomatic colonization. The decision to collect specimens for microbiological analysis was the responsibility of the attending physician that did not receive specific guidelines. We are not aware of significant changes in practice during the study period, although differences between the participating centers may exist. However, since these are expected to be minor and stable during the study period we do not feel these constitute a significant source of bias.

In this study, we found a different serotype distribution and dynamics in NIPP and IPD in the same population. This was highlighted by the fact that the potential coverage of the currently available pneumococcal vaccines with an adult indication is lower in NIPP than in IPD. The distinct dynamics of NIPP, the availability of PCV13 for adults together with the issues raised regarding the efficacy of PPV23 in the context of NIPP, and the fact that NIPP is a frequent cause of morbidity and mortality among adults, all underscore the relevance of considering the use of PCV13 in adults. However, the expected herd protection conferred by vaccinating children with PCV13 could reduce the benefits of direct adult vaccination. We documented here ongoing changes in the serotypes causing NIPP that are potentially due to long-term PCV7 use, but there is uncertainty regarding the ultimate reduction in vaccine serotypes one can expect from this effect, as well as regarding the kinetics of such a decline. Continued surveillance is essential to evaluate the changing potential benefits of direct adult vaccination.

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Author Contributions

Conceived and designed the experiments: JM-C and MR. Performed the experiments: ANH JPL and TPGSS. Analyzed the data: JM-C, MR and ANH. Contributed with reagents, materials, analysis tools: PGSSI. Contributed to the writing of the manuscript: JM-C MR ANH.

Supporting Information

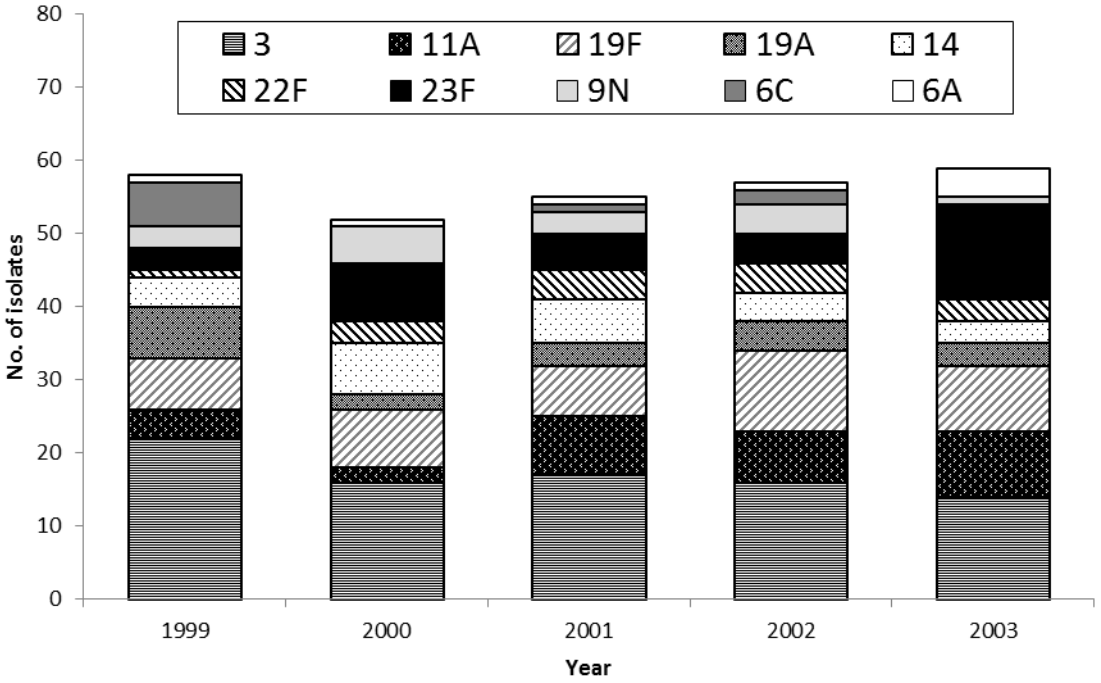

Figure IVa.S1 Serotype distribution of the isolates causing non-invasive pneumococcal pneumonia in adults in Portugal (1999–2003).

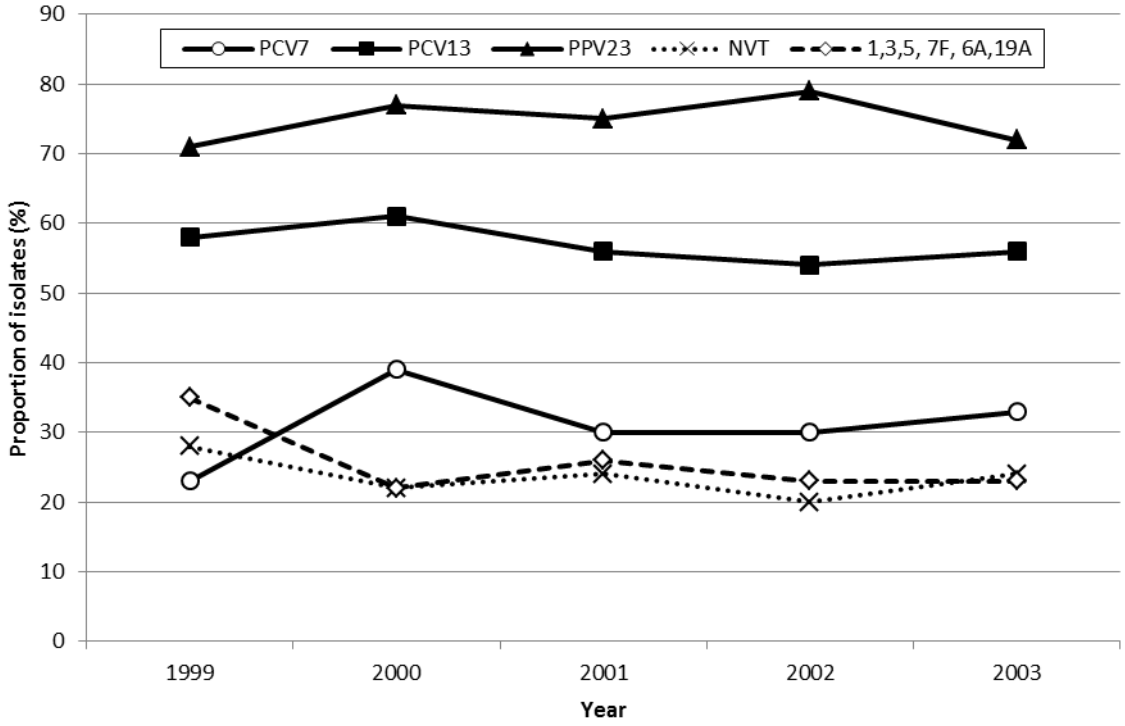

Figure IVa.S2 Proportion of isolates expressing serotypes included in pneumococcal vaccines causing non-invasive pneumococcal pneumonia in adults in Portugal (1999–2003).

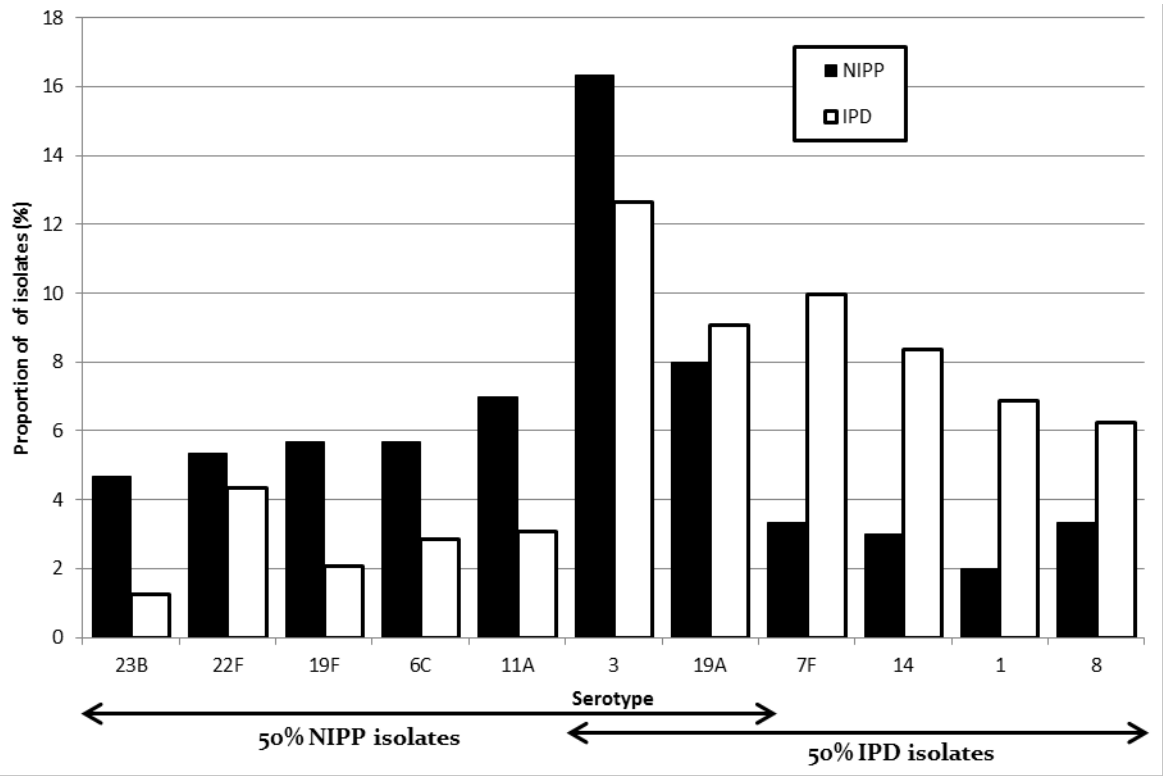

Figure IVa.S3 Proportion of isolates of each of the serotypes that together were responsible for half of non-invasive pneumococcal pneumonia isolates and half of invasive pneumococcal disease cases in adults in Portugal (2009–2011).

Table IVa.S1 Serotype distribution of the 10 most common serotypes responsible for non-invasive pneumococcal pneumonia in adults in Portugal, stratified by age groups (1999-2011).

	No. isolates (%)		
	[18-49] yrs (n=481)	[50-64] yrs (n=293)	≥65 yrs (n=526)
3	67 (13.9)	63 (21.5)	101 (19.2)
11A	29 (6.0)	16 (5.5)	42 (8.0)
19F	36 (7.5)	16 (5.5)	35 (6.7)
19A	25 (5.2)	15 (5.1)	28 (5.3)
14	20 (4.2)	13 (4.4)	20 (3.8)
22F	18 (3.7)	9 (3.1)	26 (4.9)
23F	17 (3.5)	10 (3.4)	23 (4.4)
9N	21 (4.4)	6 (2.0)	19 (3.6)
6C	16 (3.3)	8 (2.7)	13 (2.5)
6A	10 (2.1)	10 (3.4)	15 (2.9)
Other	222 (46.2)	127 (43.3)	204 (38.8)

Table IVa.S2 Isolates expressing vaccine serotypes responsible for non-invasive pneumococcal pneumonia in adults in Portugal, stratified by age groups (2009-2011).

	No. isolates (%)		
	[18-49] yrs	[50-64] yrs	≥65 yrs
PCV7	9 (12.0)	10 (12.2)	12 (8.4)
PCV13	34 (45.3)	39 (47.6)	58 (40.6)
PPV23	45 (60.0)	57 (69.5)	96 (67.1)

Table IVa.S3 Serotype distribution of the 10 overall most common serotypes in NIPP and in IPD (2009-2011).

NIPP			IPD*		
Serotype†	n (%)	Cumulative n (%)	Serotype	n (%)	Cumulative n (%)
3	49 (16.3)	49 (16.3)	3	160 (12.6)	160 (12.6)
19A	24 (8.0)	73 (24.3)	**7F**	126 (10.0)	286 (22.6)
11A	21 (7.0)	94 (31.3)	19A	115 (9.1)	401 (31.7)
6C	17 (5.7)	111 (37.0)	**14**	106 (8.4)	507 (40.1)
19F	17 (5.7)	128 (42.7)	1	87 (6.9)	594 (47.0)
22F	16 (5.3)	144 (48.0)	8	79 (6.2)	673 (53.2)
23B	14 (4.7)	158 (52.7)	22F	55 (4.3)	728 (57.5)
9N	12 (4.0)	170 (56.7)	**4**	43 (3.4)	771 (60.9)
7F	10 (3.3)	180 (60.0)	11A	39 (3.1)	810 (64.0)
8	10 (3.3)	190 (63.3)	6C	36 (2.8)	846 (66.0)

*Information published previously (19). †Serotypes in bold were associated with NIPP or IPD ($p < 0.05$).

The following study was performed by Andreia N. Horácio, Catarina Silva-Costa, Joana P. Lopes, Mário Ramirez, José Melo-Cristino and the Portuguese Group for the Study of Streptococcal Infections. Andreia N. Horácio serotyped a minor fraction of the isolates, performed most of the statistical analysis and wrote the first version of the manuscript.

CONJUGATE VACCINE SEROTYPES PERSIST AS MAJOR CAUSES OF NON-INVASIVE PNEUMOCOCCAL PNEUMONIA IN PORTUGAL DESPITE DECLINES IN SEROTYPES 3 AND 19A (2012-2015)[1]

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Abstract

Non-invasive pneumococcal pneumonia (NIPP) is a frequent cause of morbidity and mortality worldwide. The 13-valent pneumococcal conjugate vaccine (PCV13) was included in the national immunization program of children living in Portugal in 2015. Until then, PCV7 (since late 2001) and PCV13 (since early 2010) were given through the private market. We determined the serotype distribution and antimicrobial susceptibility of isolates causing adult NIPP in 2012-2015 and compared the results with previously published data (2007-2011).

There were 50 serotypes among the 1435 isolates. The most common were serotypes: 3 (14%), 11A (8%), 19F (6%), 23A (5%), 6C (5%), 19A (4%), 23B (4%), 9N (4%) and non-typable isolates (4%). When considering data since the availability of PCV13 for children in the private market, the proportion of PCV13 serotypes declined from 44.0% in 2010 to 29.7% in 2015 ($p < 0.001$), mainly due to early decreases in the proportions of serotypes 3 and 19A. In contrast, during the same period, PCV7 serotypes (11.9% in 2012-2015) and the serotypes exclusive of the 23-valent polysaccharide vaccine (26.0% in 2012-2015), remained relatively stable, while non-vaccine types increased from 27.0% in 2010 to 41.9% in 2015 ($p < 0.001$). According to the Clinical and Laboratory Standards Institute (CLSI) breakpoints, penicillin non-susceptible (PNSP) and erythromycin resistant isolates accounted for 1% and 21.7%, respectively, of the isolates recovered in 2012-2015, with no significant changes seen since 2007. Comparison of NIPP serotypes with contemporary invasive disease serotypes identified associations of 19 serotypes with either disease presentation. The introduction of PCV13 in the national immunization program for children from 2015

[1] A *facsimile* of this publication can be found at the "Publications" section of this thesis.

onwards may lead to reductions in the proportion of NIPP due to vaccine serotypes but continued NIPP surveillance is essential due to a different serotype distribution from invasive disease.

Introduction

Pneumococcal pneumonia is among the most frequent causes of death due to infection worldwide, particularly among young children and older adults (GBD 2015 Mortality and Causes of Death Collaborators, 2016). Non-invasive pneumococcal pneumonia (NIPP) is three to ten times more frequent than bacteremic pneumonia (Said et al., 2013), but studies evaluating NIPP are less abundant than those evaluating invasive pneumococcal disease (IPD).

After the introduction of pneumococcal conjugate vaccines (PCVs) for children, several studies reported a reduction of IPD in children (Pilishvili et al., 2010; Aguiar et al. 2014). Given that young children are the main reservoirs and transmitters of pneumococcus in the community and because the PCVs reduce pneumococcal colonization, several studies also reported reductions of IPD due to vaccine serotypes in the non-vaccinated population (Horácio et al., 2013; Moore et al., 2015; Waight et al., 2015; Horácio et al., 2016b).

Despite the lower number of studies, there is also evidence of herd protection in adult NIPP (Mendes et al., 2015; Rodrigo et al., 2015; Pletz et al., 2016; Georgalis et al., 2017). One study from the Netherlands suggested that, based on the similarity of vaccine serotype trends between NIPP and IPD, their national IPD data could be used to extrapolate the trends of NIPP (van Werkhoven et al., 2016). However, there are also reasons to question predictions of NIPP trends from IPD data in all settings. Perhaps the most significant is that serotype distribution and the proportion of disease that is due to vaccine serotypes differs geographically and between IPD and NIPP (Benfield et al., 2013; Horácio et al., 2014). Moreover, vaccine serotypes are free to circulate in unvaccinated people so that, especially in countries where the PCVs are not included in national immunization programs, these can persist as causes of disease, both of NIPP and IPD.

In Portugal, PCVs were available outside the national immunization program for pediatric use until mid-2015. The first PCV to become available was the 7-valent formulation (PCV7), in late-2001. Although the cost of vaccination was fully supported by the parents, the initially modest uptake of PCV7 increased steadily, reaching 75% in 2008 (Aguiar et al., 2008a). A 13-valent formulation (PCV13) replaced PCV7 in early-2010 but uptake declined, although it stayed above 60% (Silva-Costa et

al., 2018). In June 2015, PCV13 was included in the national immunization program to be given free of charge to all children born from January 2015 onwards, with a 2+1 schedule (Silva-Costa et al., 2018). Besides children, sequential vaccination with PCV13 and the 23-valent pneumococcal polysaccharide vaccine (PPV23) is recommended since 2015 by the national health authorities, but only for specific risk groups (Direção Geral da Saúde, 2015b). In addition, two Portuguese medical societies (respiratory society and general practitioner society) have issued recommendations for the sequential vaccination with PCV13 and PPV23 of all immunocompetent adults ≥ 65 years (Frões et al., 2014; Costa et al., 2016). Still, pneumococcal vaccine uptake in adults is generally believed to be low, with a study finding that < 9% of all adults ≥ 65 years had received PPV23 (Sousa et al., 2009; Fedson et al., 2011).

In a previous study we analyzed the distribution of serotypes in a randomly selected sample of 100 isolates/year collected from adult NIPP between 1999 and 2011 (Horácio et al., 2014). In the present study we aimed to gain further insights regarding vaccine serotype trends in adult NIPP in the years that followed. We characterized isolates causing adult NIPP throughout Portugal from 2012 to 2015 for serotype distribution and antimicrobial susceptibility. We also wanted to compare the NIPP data with contemporary adult IPD data obtained by the same network.

Materials and Methods

Ethics Statement

The study was approved by the Institutional Review Board of the Centro Académico de Medicina de Lisboa. These were considered surveillance activities and were exempt from informed consent. All methods were performed in accordance with the relevant guidelines and regulations. The data and isolates were de-identified so that these were irretrievably unlinked to an identifiable person.

Bacterial Isolates

Isolates were provided by a laboratory-based surveillance system that includes 30 microbiology laboratories throughout Portugal. These were asked to submit all consecutively collected pneumococci causing infections to the central laboratory. Although the laboratories were contacted periodically to submit the isolates to the central laboratory, no audit was performed to ensure compliance, which may be variable in this type of study. The identification of all isolates as *Streptococcus pneumoniae* was confirmed by colony morphology and hemolysis on blood agar plates, optochin susceptibility and bile solubility.

The isolates included in this study were recovered from sputum, bronchial secretions or bronchoalveolar lavage of adult patients (≥ 18 yrs) with a presumptive diagnosis of pneumonia between 2012 and 2015. Isolates were not included when pneumococci were simultaneously isolated from blood or another usually sterile product, and when other potential bacterial pathogens besides pneumococci were detected in the sample (such as *Haemophilus influenzae*, which was also frequently detected). Only one isolate from each patient in each year was considered.

Serotyping and Antimicrobial Susceptibility Testing

Serotyping was performed by the standard capsular reaction test using the chessboard system and specific sera (Statens Serum Institut, Copenhagen, Denmark) (Sørensen, 1993). Serotypes were classified into vaccine serotypes, i.e., those included in PCV7 (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F), in PCV13 (all PCV7 serotypes and the additional serotypes present only in PCV13, addPCV13: 1, 3, 5, 6A, 7F and 19A), in PPV23 (all PCV13 serotypes, except serotype 6A, and the additional serotypes present

only in PPV23, addPPV23: 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F) and non-vaccine serotypes (NVT, including all other serotypes). Given the high frequency of spontaneous switching between serotypes 15B and 15C we have opted to group isolates with these serotypes into a single group. Due to difficulties in phenotypically distinguishing isolates of serotype 25A and serogroup 38 and of serogroup 29 and serotype 35B these were also grouped together into the 25A/38 and 29/35B groups. The isolates that were not typable with any of the complete set of sera were considered non-typable (NT).

Minimum inhibitory concentrations (MICs) for penicillin and cefotaxime were determined using Etest strips (Biomérieux, Marcy-L'Etoile, France). Unless otherwise stated, the results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) recommended breakpoints prior to 2008 (Clinical and Laboratory Standards Institute, 2007), corresponding to the current breakpoints of oral penicillin V allowing comparison with previously published data. According to these criteria, intermediate resistance to penicillin is defined as MIC 0.12–1.0 μg/ml and high-level resistance as MIC ≥ 2.0 μg/ml. Isolates that fell into either one of these classes were designated penicillin non-susceptible (PNSP). The interpretation according to the current CLSI guidelines was also performed (Clinical and Laboratory Standards Institute, 2015). According to these criteria, for non-meningitis cases, intermediate resistance to penicillin is defined as MIC between 2–8 μg/ml and high-level resistance as MIC > 8 μg/ml. Susceptibility to cefotaxime was defined as MIC ≤ 1.0 μg/ml. The Kirby-Bauer disk diffusion assay was used to determine susceptibility to levofloxacin, erythromycin, clindamycin, chloramphenicol, trimethoprim/sulfamethoxazole, tetracycline, vancomycin and linezolid, according to the CLSI recommendations and interpretative criteria (Clinical and Laboratory Standards Institute, 2015). Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin, as previously described (Horácio et al., 2014). The MLS_B phenotype (resistance to macrolides, lincosamides and streptogramin B) was defined as the simultaneous resistance to erythromycin and clindamycin, while the M phenotype (resistance to macrolides) was defined as non-susceptibility only to erythromycin.

Statistical Analysis

Sample diversity was measured using Simpson's index of diversity (SID) and the respective 95% confidence intervals (CI95%) (Carriço et al., 2006). To compare two sets of partitions the Adjusted Wallace (AW) coefficients were calculated (Carriço et al., 2006) using the online tool available at www.comparingpartitions.info. Differences were evaluated by the Fisher exact test with the false discovery rate (FDR) correction for multiple testing (Benjamini and Hochberg, 1995) or the Chi-squared test, and the Cochran-Armitage test was used for trends. A $p < 0.05$ was considered significant for all tests.

Results

Serotype Distribution

A total of 1435 isolates were collected from adults with non-invasive pneumococcal pneumonia: n = 368 in 2012, n = 319 in 2013, n = 311 in 2014 and n = 437 in 2015. Stratifying by age group, 339 isolates (23.6%) were recovered from patients 18–49 years old, 382 (26.6%) from patients 50–64 years old and 714 (49.8%) from patients ≥ 65 years old. Most of the isolates were recovered from sputum (n = 787, 54.8%), 531 (37.0%) were recovered from bronchial secretions and 117 (8.2%) were recovered from bronchoalveolar lavage fluid. A total of 50 different serotypes were detected. The most frequent serotypes, which accounted for 52% of the isolates, were serotypes 3 (n = 196, 13.7%), 11A (n = 120, 8.4%), 19F (n = 85, 5.9%), 23A (n = 67, 4.7%), 6C (n = 64, 4.5%), 19A (n = 58, 4.0%), 23B (n = 56, 3.9%), 9N (n = 52, 3.6%) and NT isolates (n = 50, 3.5%).

The IVb.S1 Fig represent the number of isolates expressing serotypes included in PCVs, the addPPV23, and the number of isolates expressing NVTs, respectively, stratified by age group. Serotype diversity was high – SID = 0.952, CI95%: 0.948–0.956 – with no difference between SIDs of different years. No individual serotype (n > 15 isolates) showed differences in age distribution, statistically supported after FDR correction.

Fig IVb.1 shows the proportion of potentially vaccine preventable NIPP during the study period and, for comparison purposes, also the previously published data from 2007-2011, since these years represent the late post-PCV7 period (2007-2009) and the first two years of PCV13 use in children (2010-2011) (Horácio et al., 2014). Considering the evolution during the current study period only (2012–2015), there was a decline in the proportion of NIPP caused by PCV13 serotypes, from 34.5% in 2012 to 29.7% in 2015, but this was not statistically supported (p = 0.090). This decline was associated with slight and non-significant decreases in both the proportion of NIPP caused by PCV7 serotypes (from 13.6% to 11.0%, p = 0.177) and addPCV13 (from 20.9% to 18.8%, p = 0.377). In contrast, there was a non-significant increase in the proportion of NIPP caused by addPPV23 (from 24.7% in 2012 to 28.4% in 2015, p = 0.325), while the proportion of NIPP caused by NVTs remained relatively stable from 2012 to 2015

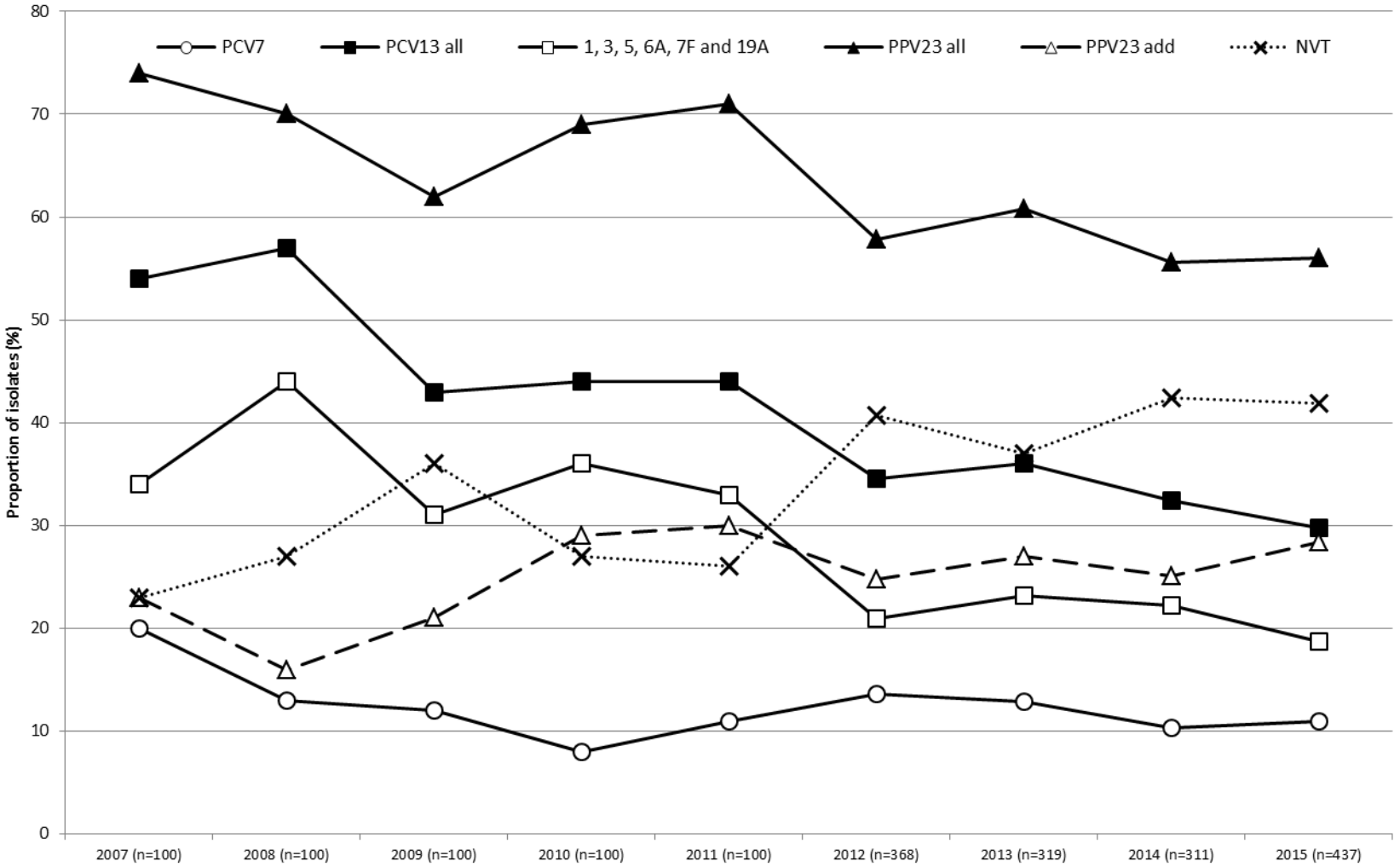

Figure IVb.1 Proportion of isolates expressing serotypes included in each of the pneumococcal vaccines causing non-invasive pneumococcal pneumonia in adult patients (≥18 years) in Portugal, 2007–2015. The data up to 2011 were presented previously (Horácio et al., 2014).

(40.8% vs 41.9%, respectively, p = 0.460).

We then evaluated possible serotype trends since 2010 when PCV13 started being used in children through the private market. The overall proportion of PCV13 serotypes declined from 44.0% in 2010 to 29.7% in 2015 (p < 0.001), while that of addPCV13 decreased from 36.0% in 2010 to 18.8% in 2015 (p < 0.001). This was accompanied by an increase of NVTs from 27.0% in 2010 to 41.9% in 2015 (p = 0.002). The PCV7 and addPPV23 serotypes remained relatively stable

Table IVb.1 shows the evolution of the individual serotypes causing NIPP in adults during the current study period (2012-2015).

Table IVb.1 Serotypes of the isolates responsible for non-invasive pneumococcal pneumonia in adult patients (≥18 years), 2012–2015.

Serotype	No. of isolates (%)				CA [a]
	2012	2013	2014	2015	2012-2015
PCV13					
1	2 (0.5)	0 (0)	0 (0)	1 (0.2)	0.399
3	48 (13.0)	54 (16.9)	41 (13.2)	53 (12.1)	0.408
4	1 (0.3)	1 (0.3)	2 (0.6)	3 (0.7)	0.329
5	0 (0)	0 (0)	0 (0)	0 (0)	-
6A	5 (1.4)	7 (2.2)	6 (1.9)	9 (2.1)	0.547
6B	7 (1.9)	4 (1.3)	4 (1.3)	6 (1.4)	0.578
7F	5 (1.4)	4 (1.3)	4 (1.3)	5 (1.1)	0.800
9V	0 (0)	5 (1.6)	0 (0)	0 (0)	0.275
14	12 (3.3)	6 (1.9)	6 (1.9)	10 (2.3)	0.426
18C	4 (1.1)	1 (0.3)	1 (0.3)	1 (0.2)	0.106
19A	17 (4.6)	9 (2.8)	18 (5.8)	14 (3.2)	0.645
19F	22 (6.0)	22 (6.9)	15 (4.8)	26 (5.9)	0.746
23F	4 (1.1)	2 (0.6)	4 (1.3)	2 (0.5)	0.483
PPV23 only					
8	7 (1.9)	10 (3.1)	6 (1.9)	16 (3.7)	0.222
9N	11 (3.0)	13 (4.1)	16 (5.1)	12 (2.7)	0.942
10A	11 (3.0)	6 (1.9)	5 (1.6)	10 (2.3)	0.519
11A	29 (7.9)	29 (9.1)	22 (7.1)	40 (9.2)	0.703
12F	0 (0)	0 (0)	0 (0)	0 (0)	-
15B/C	6 (1.6)	11 (3.4)	7 (2.3)	10 (2.3)	0.807
17F	8 (2.2)	4 (1.3)	8 (2.6)	4 (0.9)	0.319
20	5 (1.4)	5 (1.6)	6 (1.9)	8 (1.8)	0.557
22F	14 (3.8)	8 (2.5)	6 (1.9)	21 (4.8)	0.448
33F	0 (0)	0 (0)	2 (0.6)	3 (0.7)	0.048
NVT [b]					
6C	19 (5.2)	16 (5.0)	7 (2.3)	22 (5.0)	0.627
23A	24 (6.5)	12 (3.8)	14 (4.5)	17 (3.9)	0.130
23B	15 (4.1)	8 (2.5)	15 (4.8)	18 (4.1)	0.631
NT	9 (2.4)	8 (2.5)	16 (5.1)	17 (3.9)	0.123
15A	10 (2.7)	9 (2.8)	8 (2.6)	16 (3.7)	0.465
31	15 (4.1)	7 (2.2)	14 (4.5)	12 (2.7)	0.587
16F	7 (1.9)	10 (3.1)	3 (1.0)	20 (4.6)	0.070

Table IVb.1 (continued).

Serotype	No. of isolates (%)				CA [a]
	2012	2013	2014	2015	2012-2015
NVT[b]					
29/35B	12 (3.3)	6 (1.9)	6 (1.9)	10 (2.3)	0.426
35F	5 (1.4)	3 (0.9)	6 (1.9)	14 (3.2)	0.033
34	3 (0.8)	8 (2.5)	5 (1.6)	8 (1.8)	0.445
21	3 (0.8)	6 (1.9)	7 (2.3)	8 (1.8)	0.265
24F	4 (1.1)	2 (0.6)	6 (1.9)	10 (2.3)	0.082
33A	6 (1.6)	1 (0.3)	5 (1.6)	0 (0)	0.052
25A/38	7 (1.9)	2 (0.6)	0 (0)	0 (0)	**0.001**
35A	2 (0.5)	3 (0.9)	4 (1.3)	2 (0.5)	0.946
7C	2 (0.5)	2 (0.6)	6 (1.9)	1 (0.2)	0.946
13	2 (0.5)	3 (0.9)	1 (0.3)	1 (0.2)	0.333
37	1 (0.3)	4 (1.3)	1 (0.3)	3 (0.7)	0.802
Others [c]	4 (1.1)	8 (2.5)	8 (2.6)	4 (0.9)	-
Total	368	319	311	437	-

[a]CA, Cochran Armitage test of trend. In bold is the only serotype with significant p-value ($p < 0.05$) after FDR correction. [b]NVT, non-vaccine serotypes, i.e., serotypes not included in any of the currently available pneumococcal vaccines. [c]Only serotypes detected in ≥3 isolates in at least one year are shown; the remaining are grouped together under "Others."

Only serotype 25A/38 significantly changed its proportion after FDR correction (from 1.9% in 2012 to 0.0% in 2015, $p = 0.001$). When considering the evolution of individual serotypes since 2007 (Table IVb.1 and Table IVb.S1), only four serotypes significantly changed their proportion after FDR correction (Fig IVb.S2), which were serotypes 3 (declined from 22.0% in 2007 to 12.1% in 2015, $p < 0.001$), 19A (declined from 6.0% in 2007 to 3.2% in 2015, $p = 0.003$), 7F (declined from 3.0% in 2007 to 1.1% in 2015, $p = 0.004$) and 35F (increased from 0% in 2007 to 3.2% in 2015, $p = 0.003$). The declines in proportion of serotypes 3 and 19A showed important yearly fluctuations and these were also found for several other serotypes (Table IVb.1 and Table IVb.S1).

When analyzing the evolution of individual vaccine serotypes and of vaccine serotype groups in 2012-2015 stratified by age group (Table IVb.2), there were no significant changes after FDR correction. When considering data since 2007, only for serotype 3 and for adults aged ≥ 65 years old did the change remain statistically supported after FDR correction (serotype 3 declined from 27.5% in 2007 to 11.1% in 2015, $p < 0.001$).

Table IVb.2 Number of isolates responsible for non-invasive pneumococcal pneumonia in adult patients (≥18 years), according to vaccine serotype groups and age groups, 2012–2015.

	Serotype Groups[b]	No. isolates (%)				C. A.[a]
		2012	2013	2014	2015	
18-49 yrs	PCV7	12 (12.9)	11 (16.4)	8 (10.3)	16 (15.8)	0.782
	1, 5 and 7F	1 (1.1)	1 (1.5)	2 (2.6)	1 (1.0)	0.926
	3, 6A and 19A	19 (20.4)	11 (16.4)	10 (12.8)	20 (19.8)	0.800
	PCV13	32 (34.4)	23 (34.3)	20 (25.6)	37 (36.6)	0.983
	PPV23 add	25 (26.9)	15 (22.4)	28 (35.9)	30 (29.7)	0.417
	NVTs	36 (38.7)	29 (43.3)	30 (38.5)	34 (33.7)	0.385
50-64 yrs	PCV7	12 (14.1)	7 (8.4)	7 (7.4)	8 (6.7)	0.328
	1, 5 and 7F	3 (3.5)	0 (0)	1 (1.1)	1 (0.8)	0.656
	3, 6A and 19A	15 (17.6)	23 (27.7)	23 (24.2)	19 (16.0)	0.095
	PCV13	30 (35.3)	30 (36.1)	31 (32.6)	28 (23.5)	0.026
	PPV23 add	45 (18.8)	50 (27.7)	53 (24.2)	65 (32.8)	0.082
	NVTs	39 (45.9)	30 (36.1)	41 (24.2)	52 (32.8)	0.531
≥65 yrs	PCV7	26 (13.7)	23 (13.6)	17 (12.3)	24 (11.1)	0.381
	1, 5 and 7F	3 (1.6)	3 (1.8)	1 (0.7)	4 (1.8)	0.976
	3, 6A and 19A	36 (18.9)	36 (21.3)	32 (23.2)	37 (17.1)	0.665
	PCV13	65 (34.2)	62 (36.7)	50 (36.2)	65 (30.0)	0.332
	PPV23 add	50 (26.3)	48 (28.4)	27 (19.6)	55 (25.3)	0.078
	NVTs	75 (39.5)	59 (34.9)	61 (44.2)	97 (44.7)	0.125

[a]CA, Cochran Armitage test of trend. [b]PCV7, serotypes included in the 7-valent pneumococcal conjugate vaccine. PCV13, serotypes included in the 13-valent pneumococcal conjugate vaccine. addPPV23, the additional 11 serotypes present in the 23-valent pneumococcal polysaccharide vaccine but absent from PCV13. NVTs, serotypes not included in any of the currently available pneumococcal vaccines.

Antimicrobial Susceptibility

Susceptibility to the tested antimicrobials between 2012 and 2015 stratified by the age groups considered is summarized in Table IVb.3. When considering all isolates, a total of n = 258 isolates (18.0%) were classified as PNSP of which n = 229 (88.8%) expressed low-level resistance and n = 29 (11.2%), high-level resistance. According to the current CLSI guidelines for parental penicillin in non-meningitis cases (Clinical and Laboratory Standards Institute, 2015), only n = 15 isolates (1.0%) would have been considered PNSP, with only 2 of these expressing high-level resistance. A total of n = 311 isolates (21.7%) were classified as erythromycin resistant pneumococci (ERP). Of these, n = 246 isolates (79.1%) expressed the MLS_B phenotype, while the remaining (n = 65, 20.9%) presented the M phenotype. A total of 12.3% (n =

176) of the isolates were simultaneously non-susceptible to penicillin and resistant to erythromycin (EPNSP).

There were no significant variations in antimicrobial resistance during the current study period (2012-2015), nor were there significant changes in antimicrobial resistance when considering NIPP from 2007 (Horácio et al., 2014). Although with moderate overall AW values [the AW for serotype to PNSP was 0.441 (CI95%: 0.386-0.496) and the AW for serotype to ERP was 0.443 (CI95%: 0362-0.524)], there was an association between certain serotypes and antimicrobial resistance (Fig IVb.S1). The serotypes that were positively associated with PNSP after FDR correction were serotypes 6C, 14, 15A, 19F, 19A and 23F. Among these, serotypes 19F (15.5%), 14 (12.4%), 6C (12.0%) and 19A (11.6%) accounted for half of all PNSP. The serotypes which were positively associated with ERP after FDR correction were serotypes 6B, 6C, 14, 15A,19F, 19A, 33A and 35A, of which serotypes 19F (20.3%), 19A (10.6%), 6C (9.6%) and 15A (8.0%) accounted for half of all ERP. The PCV7, PCV13 and PPV23 serotypes accounted for 33.7%, 47.3% and 53.5% of PNSP, respectively, and 33.4%, 49.5% and 54.3% of ERP, respectively.

Table IVb.3 Antimicrobial resistance of the isolates responsible for non-invasive pneumococcal pneumonia in adult patients (≥18 years) in Portugal, 2012–2015.

	No. resistant isolates (%)[a]		
	18-49 years (n = 339)	**50-64 years (n = 382)**	**≥65 years (n = 714)**
PEN[b]	57 (16.8)	70 (18.3)	131 (18.3)
MIC_{90}	0.19	0.19	0.38
MIC_{50}	0.012	0.012	0.012
CTX	7 (2.1)	7 (1.8)	4 (0.6)
MIC_{90}	0.25	0.25	0.38
MIC_{50}	0.015	0.016	0.016
LEV	2 (0.6)	3 (0.8)	16 (2.2)
ERY	73 (21.5)	70 (18.3)	168 (23.5)
CLI	56 (16.5)	58 (15.2)	136 (19.0)
CHL	7 (2.1)	7 (1.8)	4 (0.6)
SXT	57 (16.8)	66 (17.3)	104 (14.6)
TET	59 (17.4)	55 (14.4)	115 (16.1)

[a]PEN, penicillin; CTX, cefotaxime; LEV, levofloxacin; ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline. All isolates were susceptible to vancomycin and linezolid. [b]Non-susceptibitily to penicillin was determined using the CLSI breakpoints prior to 2008 (Clinical and Laboratory Standards Institute, 2007).

Discussion

The present study documented a decline of PCV13 serotypes in adult NIPP in the post-PCV13 period. This occurred mostly in 2011-2012 but continued, albeit more moderately, in recent years, from 44.0% in 2010 to 29.7% in 2015. It was also noted that during 2007-2015 there were several important yearly fluctuations in the proportion of individual serotypes, both among PCV13 and non-PCV13 serotypes (Table IVb.1 and Table IVb.S1). This suggests that variations of the PCV13 serotypes in the post-PCV13 period in adult NIPP could be the result of, not only the herd protection conferred by childhood vaccination with PCV13, but also of temporal trends, which had been documented in adult NIPP in Portugal previously (Horácio et al., 2014).

The evolution of PCV13 serotypes in adult NIPP from 2010 onwards (when PCV13 was being used for children vaccination in the private market) was different from the one previously found for adult IPD in Portugal in a similar period (Horácio et al., 2016b). While in NIPP the sharpest decrease in addPCV13 serotypes in the post-PCV13 period occurred from 2011 to 2012, in IPD this occurred only from 2012 to 2013. Although the decrease of addPCV13 serotypes in adult NIPP may have been also influenced by temporal trends, the sustained lower values found from 2013 onwards suggest an important contribution of herd protection resulting from PCV13 childhood vaccination.

Serotypes 3 and 19A had a major influence in the decrease of PCV13 serotypes in adult NIPP in the post-PCV13 period (2010-2015), although these changes were also most significant in the first years (Table IVb.1 and Table IVb.S1). Similarly, in adult IPD two serotypes accounted for most of the decline in the prevalence of PCV13 serotypes in the post-PCV13 period, but in this case these were serotypes 7F and 19A (Horácio et al., 2016b). In contrast with the declines of serotypes 3 and 19A in adult NIPP, the decreases of serotypes 7F and 19Ain adult IPD were more pronounced and sustained.

Serotype 3 has been the dominant serotype in adult NIPP and IPD in Portugal, both before and after the introduction of PCV13 for children (Horácio et al., 2014; Horácio et al., 2016b). The decline in serotype 3 in NIPP is surprising because this serotype did not show major changes in adult IPD in the post-PCV13 period in

Portugal (Horácio et al., 2016b) nor in other countries (Steens et al., 2013; Harboe et al., 2014; Moore et al., 2015; Waight et al., 2015; Ladhani et al., 2018). However, reductions in incidence of serotype 3 NIPP was reported in other studies, including a study from England (Rodrigo et al., 2015). The reduced efficacy of PCV13 in preventing pediatric complicated pneumonias caused by serotype 3 (Silva-Costa et al., 2018) and the use of PCV13 outside of the national immunization program with somewhat modest uptake, raise the possibility of continued circulation of this serotype in carriage, potentially explaining its persistence in disease. Since serotype 3 is heterogeneous in its invasive disease potential, meaning that there are different clones expressing this serotype that differ in their capacity to cause invasive disease (Sá-Leão et al., 2011), it is possible that more invasive clones of serotype 3 have increased post-PCV use for reasons that remain unknown. In adult IPD, there was an expansion of the multilocus sequence type clonal complex CC180 among isolates expressing serotype 3 (Horácio et al., 2016a), prior to the use of PCV13 in children, but no information is available in the post-vaccine period.

Serotype 19A emerged in Portugal in the late post-PCV7 period, to become one of the most important serotypes in both adult NIPP (Horácio et al., 2014) and IPD (Horácio et al., 2012; Horácio et al., 2013; Horácio et al., 2016b). A decrease of serotype 19A in adult IPD and in NIPP in the post-PCV13 period was documented not only for Portugal but for other countries (Mendes et al., 2015; Moore et al., 2015; Rodrigo et al., 2015; Waight et al., 2015; Ladhani et al., 2018). Given the compelling evidence of herd protection in adult IPD resulting from PCV13 use in children in serotype 19A, the lack of a more significant reduction of serotype 19A in adult NIPP in the post-PCV13 period could be due to a particular propensity of this serotype to cause NIPP. A clearer picture of the impact of PCV13 use in children in reducing the importance of serotypes 3 and 19A in adult NIPP may only be provided by further studies following the epidemiology of adult NIPP after the inclusion of PCV13 in the national immunization plan.

A decrease in serotype 7F was also detected but its contribution to the reduction of PCV13 serotypes in NIPP was minor since this serotype was an uncommon cause of NIPP in the pre-PCV13 period.

Contrasting with the declining trend of PCV13 serotypes, no significant trend was seen for PCV7 serotypes in adult NIPP and this was mostly due to the persistence of serotype 19F, which occurred in 49% of the isolates expressing a PCV7 serotype in 2012-2015. Despite being targeted by all PCVs available to date, serotype 19F remained common in nasopharyngeal carriage of children in Portugal in the late post-PCV7 period (Horácio et al., 2012) and in the post-PCV13 period (Rodrigues et al., 2013) including among vaccinated children. The inability of the PCVs to eliminate this serotype from carriage in children, at least in a non-universal coverage scenario, together with its likely intrinsic propensity to cause NIPP rather than IPD (Horácio et al., 2014) as was also shown here, may have contributed to why this serotype remained the third most frequent cause of adult NIPP in the post-PCV13 period in Portugal.

The decrease of PCV13 serotypes in the post-PCV13 period was accompanied by an increase in the proportion of NVTs, while the addPPV23 serotypes remained relatively stable. However, among the NVTs, only one serotype was clearly emerging (serotype 35F) and only in the last year of the study period. Most of the remaining increase in NVTs was based in increases in the proportion of serotypes 16F, 24F and NTs (Table IVb.1 and Table IVb.S1), which were not significant if considered independently. This contrasts with results from adult IPD, in which there were several non-PCV13 emerging serotypes (serotypes 8, 22F, 20 and 15A), most of them included in PPV23 (Horácio et al., 2016b). These differences are not surprising, since isolates responsible for adult NIPP and adult IPD are known to have different serotype distributions (Benfield et al., 2013; Horácio et al., 2014).

When comparing the serotype distribution of the isolates causing adult NIPP in 2012-2014 n 2012–2014 with the serotype distribution of isolates causing adult IPD in the same period (Table IVb.S2), serotypes 11A, 19F, 23A, 23B, 31, NT, 17F, 6A, 21 and 37 (ranked by their frequency in NIPP) were significantly associated with NIPP, while serotypes 8, 19A, 22F, 14, 7F, 20, 1, 4 and 12B (ranked by their frequency in IPD) were significantly associated with IPD. Most of these associations had been already recognized in the pre-PCV13 period (Horácio et al., 2014), while the new associations in adult IPD reflect mainly the emerging serotypes in the post-PCV13 period.

While antimicrobial resistance declined in adult IPD in the post-PCV13 period, no decline was found for adult NIPP in this study. In NIPP, the small decrease in proportion of the mostly antimicrobial resistant serotype 19A isolates, was balanced by an increase of NT isolates, which were also associated with antimicrobial resistance. NTs were found to be frequent colonizers of the nasopharynx of children in the post-PCV13 period (Rodrigues et al., 2013) and were more frequently found in NIPP than in IPD (Table IVb.S2). The stability of PCV7 serotypes in the post-PCV13 period also helped maintaining antimicrobial resistance rates in adult NIPP (Horácio et al., 2014).

The study presented has the limitations discussed previously (Horácio et al., 2014). These include the possibility that some of the isolates we identified as being responsible for NIPP were in fact causing bacteremic pneumonia or reflected colonization and not disease. Despite the general recommendation that both blood and respiratory tract samples should be collected for the etiologic diagnosis of pneumonia, we cannot guarantee that this was done in all cases. However, we consider these to account, at most, for a small fraction of the isolates and therefore not to introduce a significant bias. Moreover, the distinct serotype distribution found in this study for IPD and NIPP, strongly argues against this possibility. Since our study is laboratory-based, it was not designed to collect information important to assess the severity of the infections caused by the different serotypes (e.g. hospitalization, ICU admission, 30-day mortality). However, this does not compromise our approach of comparing the serotype distribution of IPD and NIPP cases. Our temporal analyses were based on previously published data reporting the characteristics of a random sample of 100 isolates per year (Horácio et al., 2014). Since not all available isolates before 2012 were characterized, it is possible that some of the changes in the serotype distribution occurring from 2011 to 2012 are due to this sampling process.

In this study it was found that the overall proportion of PCV13 serotypes decreased only moderately in adult NIPP in the post-PCV13 period. In 2015, 30% of NIPP was due to PCV13 serotypes and 28% was due to the addPPV23 serotypes, highlighting the potential role of vaccination in disease prevention. However, the inclusion of PCV13 in the national immunization program for children in 2015 and the anticipated declines in at least some of the PCV13 serotypes due to herd effect, raise

important issues regarding the cost-effectiveness of a universal adult vaccination program. However, because the magnitude and timeframe of this herd effect remains poorly defined, particularly in NIPP, further surveillance is essential to document future trends in pneumococcal serotype prevalence in adult NIPP, as these seem to differ from adult IPD.

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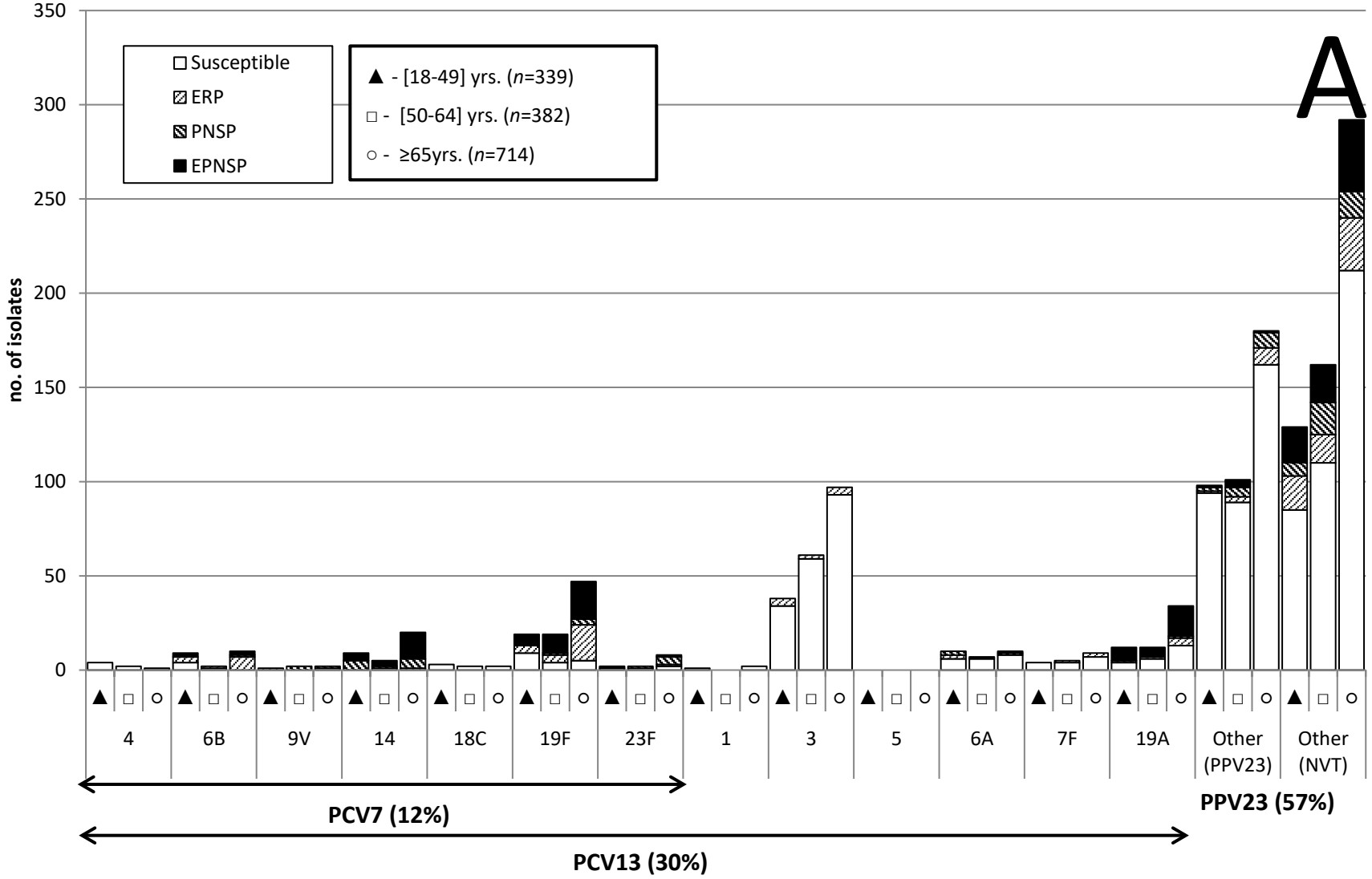
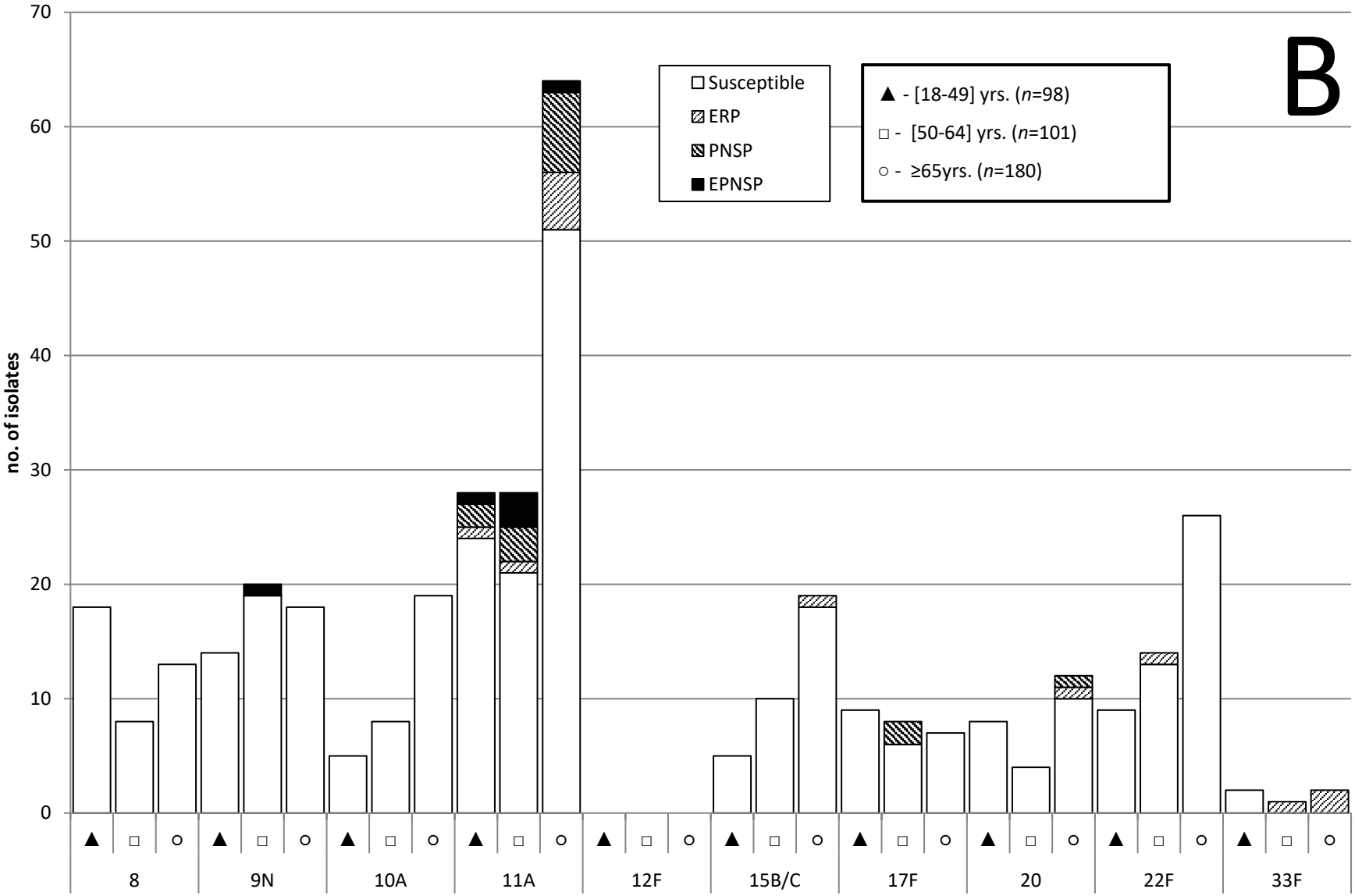
Supporting Information




B
no. of isolates
70
60
50
40
30
20
10
0
Susceptible
ERP
PNSP
EPNSP
▲ - [18-49] yrs. (n=98)
□ - [50-64] yrs. (n=101)
○ - ≥65yrs. (n=180)
8
9N
10A
11A
12F
15B/C
17F
20
22F
33F

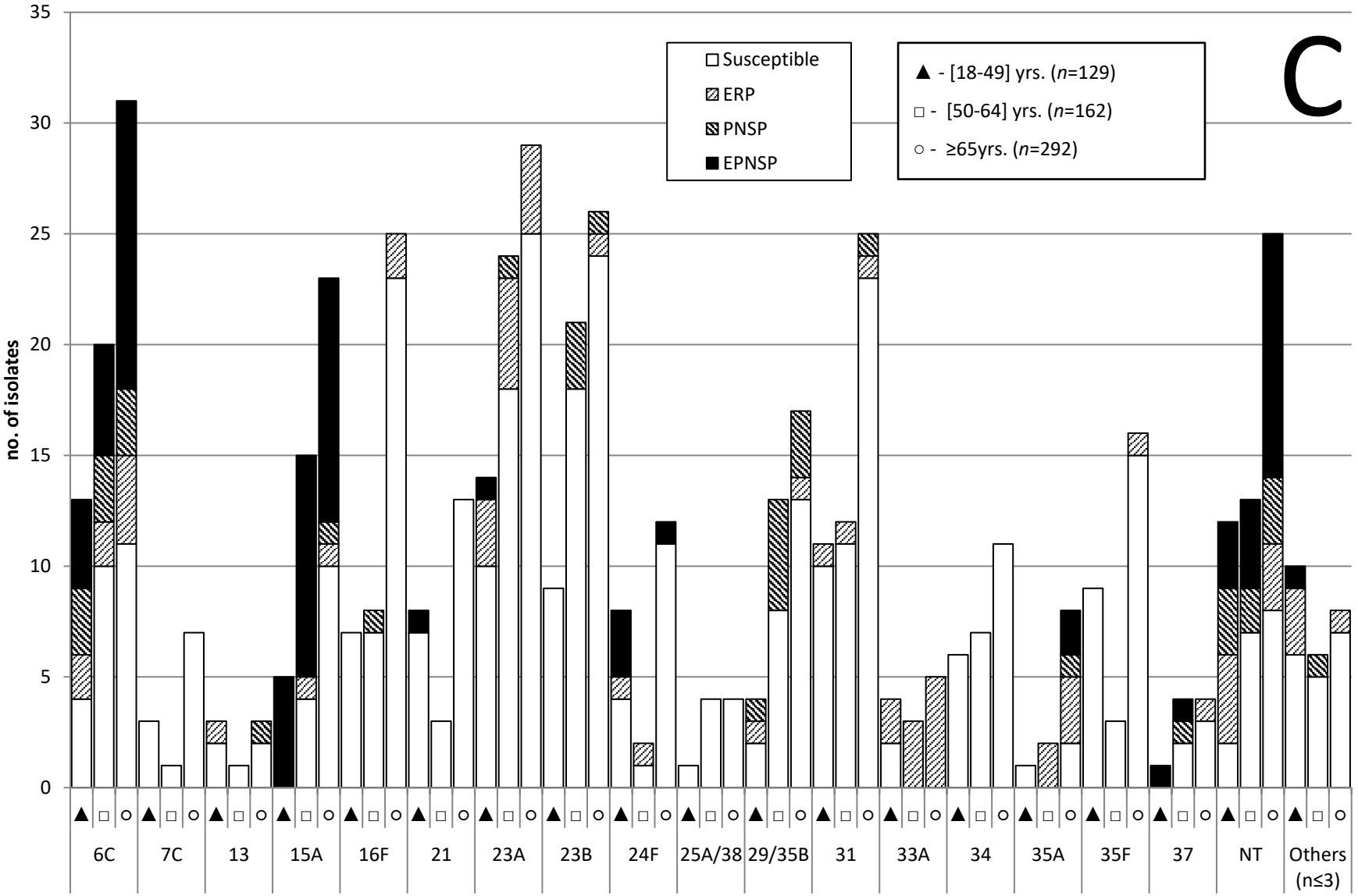

C
no. of isolates
35
30
25
20
15
10
5
0
Susceptible
ERP
PNSP
EPNSP
▲ - [18-49] yrs. (n=129)
□ - [50-64] yrs. (n=162)
○ - ≥65yrs. (n=292)
6C
7C
13
15A
16F
21
23A
23B
24F
25A/38
29/35B
31
33A
34
35A
35F
37
NT
Others (n≤3)

Fig IVb.S1. Number of isolates expressing each serotype causing non-invasive pneumococcal pneumonia in adult patients (≥18 yrs), Portugal, 2012-2015. The number of isolates expressing each serotype in each of the age groups considered is indicated. Isolates recovered from patients 18-49 years are indicated by black triangles. Isolates recovered from patients 50-64 years are indicated by open squares. Isolates recovered from patients ≥ 65years are indicated by open circles. Isolates presenting both erythromycin resistance and penicillin non-susceptibility (EPNSP) are represented by closed black bars. Penicillin non-susceptible isolates (PNSP) are indicated by dark hatched bars. Erythromycin resistant pneumococci (ERP) are indicated by light hatched bars. Isolates susceptible to both penicillin and erythromycin are represented by white open bars. Panel A - Serotypes included in conjugate vaccines. The serotypes included in the seven-valent conjugate vaccine (PCV7) and in the 13-valent conjugate vaccine (PCV13) are indicated by the arrows. NVT, non-vaccine serotypes; addPPV23, the additional serotypes included in the 23-valent polysaccharide vaccine but not included in PCV13. Panel B - Additional serotypes included in the 23-valent polysaccharide vaccine but not included in the 13-valent conjugate vaccine. Out of the 11 addPPV23 serotypes only serotype 2 was not found in our collection. Panel C - Serotypes not included in any pneumococcal vaccine. NT, non-typable. Isolates expressing serotypes 25A and 38 and serotypes 29 and 35B could not be distinguished phenotypically and are represented together. Only serotypes including n>3 isolates are discriminated, all remaining serotypes are grouped together under the “Others” category grouping isolates of serotypes: 10B, 12B, 17A, 18A (n=3 each); 10F, 11F, 11B and 47F (n=2 each) and 28A, 35C, 36 and 42 (n=1 each).

Table IVb.S1: Serotypes of the isolates responsible for non-invasive pneumococcal pneumonia in adult patients (≥18 years), 2007–2011. These data were presented previously (Horácio et al., 2014).

Serotype[a]	No. of isolates					CA[b]
	2007	2008	2009	2010	2011	2007-2015
PCV13						
1	0	0	3	2	1	0.039
3	22	28	9	22	18	**<0.001**
4	1	0	0	1	1	0.776
5	0	0	2	0	0	0.041
6A	3	3	2	4	3	0.342
6B	3	1	0	1	0	0.926
7F	3	4	4	4	2	**0.004**
9V	3	0	0	0	0	0.048
14	4	3	4	2	3	0.172
18C	0	2	0	0	0	0.435
19A	6	9	11	4	9	**0.003**
19F	6	3	7	4	6	0.682
23F	3	4	1	0	1	0.011
PPV23 only						
8	3	0	3	2	5	0.316
9N	1	0	4	6	2	0.184
10A	2	0	2	1	1	0.394
11A	9	8	4	10	7	0.582
12F	0	0	0	0	1	0.555
15B/C	1	2	4	3	3	0.864
17F	1	2	0	0	3	0.734
20	0	1	1	0	2	0.087
22F	6	3	3	7	6	0.398
33F	0	0	0	0	0	0.035
NVT						
6C	2	4	10	5	2	0.786
23A	3	4	3	1	2	0.440
23B	2	2	4	3	7	0.308
NT	1	3	2	1	2	0.021
15A	2	3	4	2	4	0.632
31	2	1	1	1	0	0.063
16F	2	2	2	3	0	0.097
29/35B	2	1	2	4	2	0.974
35F	0	0	1	1	1	**0.003**
34	0	0	0	1	2	0.023
21	0	0	1	1	0	0.015
24F	2	0	2	0	0	0.133
33A	0	1	2	1	1	0.373
25A/38	1	2	1	0	0	0.024
35A	0	1	0	0	0	0.305
7C	0	0	0	0	0	0.102
13	1	0	0	1	1	0.600
37	0	0	0	0	0	0.102
Others[c]	3	3	1	2	2	-
Total	100	100	100	100	100	-

[a]NVT, non-vaccine serotypes, i.e., serotypes not included in any of the currently available pneumococcal vaccines. [b]CA, Cochran Armitage test of trend. In bold are the serotypes with significant p-value ($p < 0.05$) after FDR correction. [c]Only serotypes detected in ≥3 isolates or in at least 2 years are shown; the remaining are grouped together under "Others."

Table IVb.S2: Serotype distribution of the isolates causing non-invasive pneumococcal pneumonia and invasive pneumococcal disease in adults in Portugal (2012–2014).

Serotype	n (%)		OR [b]	$CI_{95\%}$	p value [c]
	NIPP (n=998)	IPD (n=1163) [a]			
3	143 (14.3)	161 (13.8)	1.1	(0.8-1.3)	0.757
11A	80 (8.0)	49 (4.2)	2.0	(1.4-2.9)	**<0.001**
19F	59 (5.9)	27 (2.3)	2.6	(1.6-4.4)	**<0.001**
23A	50 (5.0)	26 (2.2)	2.3	(1.4-3.9)	**0.001**
19A	44 (4.4)	84 (7.2)	0.6	(0.4-0.9)	**0.006**
6C	42 (4.2)	28 (2.4)	1.8	(1.1-3.0)	0.020
9N	40 (4.0)	39 (3.4)	1.2	(0.7-1.9)	0.424
23B	38 (3.8)	12 (1.0)	3.8	(1.9-8.0)	**<0.001**
31	36 (3.6)	11 (0.9)	3.9	(1.9-8.6)	**<0.001**
NT	33 (3.3)	10 (0.9)	3.9	(1.9-9.0)	**<0.001**
22F	28 (2.8)	79 (6.8)	0.4	(0.2-0.6)	**<0.001**
15A	27 (2.7)	27 (2.3)	1.2	(0.7-2.1)	0.583
14	24 (2.4)	73 (6.3)	0.4	(0.2-0.6)	**<0.001**
15B/C	24 (2.4)	22 (1.9)	1.3	(0.7-2.4)	0.456
29/35B	24 (2.4)	26 (2.1)	1.1	(0.6-2.0)	0.886
8	23 (2.3)	123 (10.6)	0.2	(0.1-0.3)	**<0.001**
10A	22 (2.2)	18 (1.5)	1.4	(0.7-2.9)	0.267
16F	20 (2.0)	23 (2.0)	1.0	(0.5-1.9)	1
17F	20 (2.0)	9 (0.8)	2.6	(1.1-6.6)	**0.015**
6A	18 (1.8)	7 (0.6)	3.0	(1.2-8.6)	**0.014**
20	16 (1.6)	39 (3.4)	0.5	(0.2-0.9)	**0.009**
21	16 (1.6)	0 (0)	Inf	(4.5-inf)	**<0.001**
34	16 (1.6)	8 (0.7)	2.4	(0.9-6.4)	0.062
6B	15 (1.5)	15 (1.3)	1.2	(0.5-2.6)	0.715
35F	14 (1.4)	13 (1.1)	1.3	(0.5-2.9)	0.566
7F	13 (1.3)	61 (5.2)	0.2	(0.1-0.4)	**<0.001**
24F	12 (1.2)	23 (2.0)	0.6	(0.3-1.3)	0.173
33A	12 (1.2)	9 (0.8)	1.6	(0.6-4.2)	0.381
7C	10 (1.0)	6 (0.5)	2.0	(0.6-6.6)	0.215
23F	10 (1.0)	13 (1.1)	0.9	(0.3-2.2)	0.836
25A/38	9 (0.9)	8 (0.7)	1.3	(0.4-3.9)	0.631
35A	9 (0.9)	2 (0.2)	5.3	(1.1-50.3)	0.029
13	6 (0.6)	2 (0.2)	3.5	(0.6-35.6)	0.155
18C	6 (0.6)	7 (0.6)	1.0	(0.3-3.5)	1
37	6 (0.6)	0 (0)	Inf	(1.4-inf)	**0.010**
9V	5 (0.5)	9 (0.8)	0.6	(0.2-2.2)	0.593
4	4 (0.4)	23 (2.0)	0.2	(0.1-0.6)	**0.001**
12B	3 (0.3)	18 (1.5)	0.2	(0-0.7)	**0.003**
17A	3 (0.3)	0 (0)	Inf	(0.5-inf)	0.098
1	2 (0.2)	26 (2.2)	0.1	(0-0.4)	**<0.001**
10B	2 (0.2)	0 (0)	Inf	(0.2-inf)	0.213
10F	2 (0.2)	1 (0.1)	2.3	(0.1-137.7)	0.599
11B	2 (0.2)	2 (0.2)	1.2	(0.1-16.1)	1
18A	2 (0.2)	3 (0.3)	0.8	(0.1-6.8)	1
33F	2 (0.2)	3 (0.3)	0.8	(0.1-6.8)	1
47F	2 (0.2)	1 (0.1)	2.3	(0.1-137.7)	0.599
28A	1 (0.1)	1 (0.1)	1.2	(0-91.5)	1
35C	1 (0.1)	0 (0)	Inf	(0-inf)	0.462

Table IVb.S2 (continued).

Serotype	n (%)		OR [b]	$CI_{95\%}$	p value [c]
	NIPP (n=998)	IPD (n=1163) [a]			
36	1 (0.1)	0 (0)	Inf	(0-inf)	0.462
42	1 (0.1)	0 (0)	Inf	(0-inf)	0.462
5	0 (0)	1 (0.1)	0	(0-45.4)	1
6D	0 (0)	1 (0.1)	0	(0-45.4)	1
12A	0 (0)	2 (0.2)	0	(0-6.2)	0.503
16A	0 (0)	2 (0.2)	0	(0-6.2)	0.503
18F	0 (0)	2 (0.2)	0	(0-6.2)	0.503
19B	0 (0)	1 (0.1)	0	(0-45.4)	1
22A	0 (0)	1 (0.1)	0	(0-45.4)	1
24A	0 (0)	2 (0.2)	0	(0-6.2)	0.502
24B	0 (0)	1 (0.1)	0	(0-45.4)	1
33B	0 (0)	2 (0.2)	0	(0-6.2)	0.503
39	0 (0)	1 (0.1)	0	(0-45.4)	1

[a]IPD - invasive pneumococcal disease. NIPP – non-invasive pneumococcal pneumonia. Data from IPD were published previously (Horácio et al., 2016b). [b]Odds ratios and 95% confidence intervals (CI95%) were used to measure the association between serotype and disease presentation. [c]In bold are significant p-values ($p < 0.05$) after FDR correction.

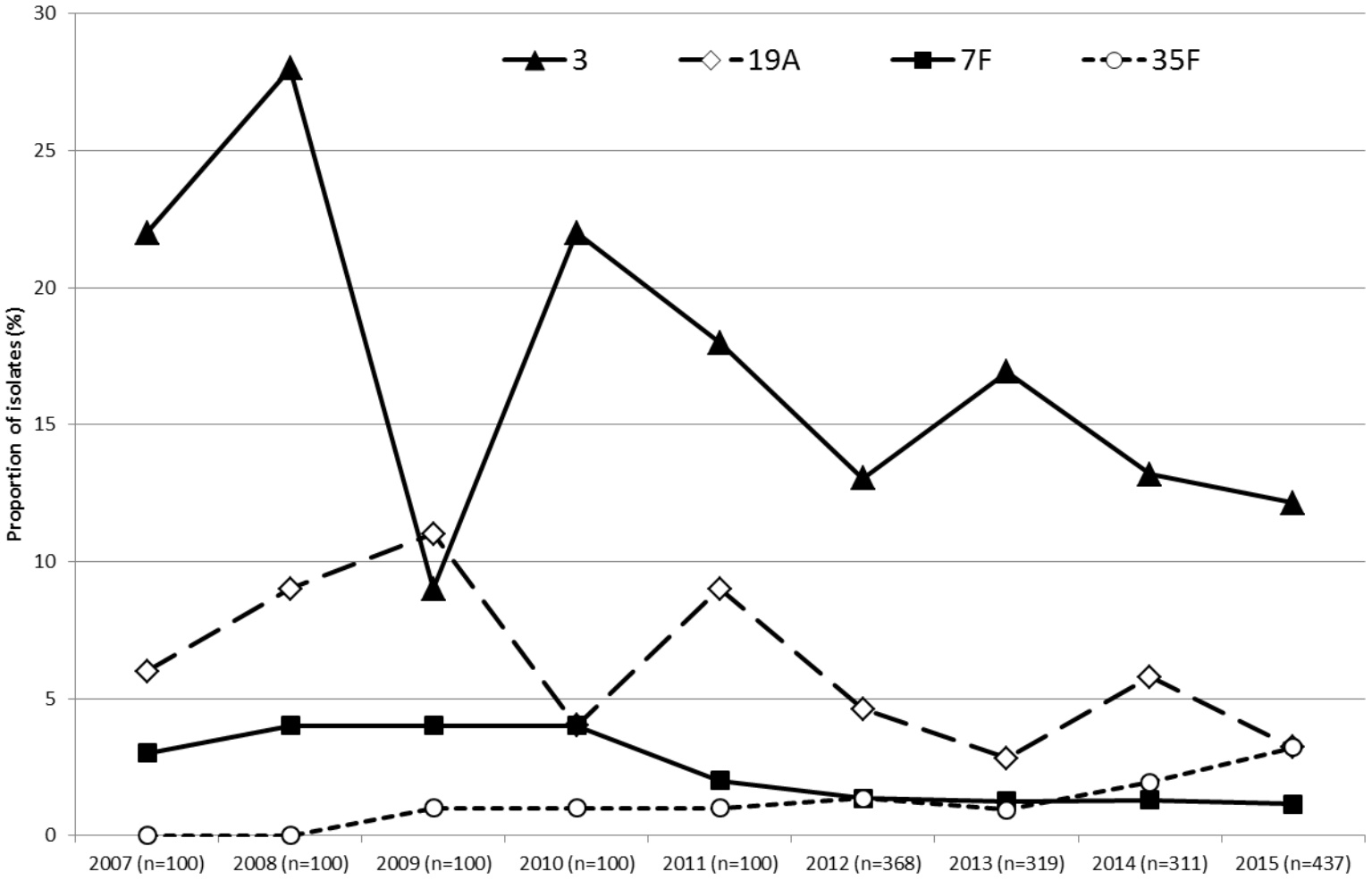


Figure IVb.S2 Proportion of isolates expressing serotypes that changed in proportion after FDR correction causing non-invasive pneumococcal pneumonia in adult patients (≥18 years) in Portugal, 2007–2015. The data up to 2011 were presented previously (Horácio et al., 2014).

CHAPTER V – GENERAL DISCUSSION

The high morbidity and mortality associated with pneumococcal infections boosted a search for vaccines that could prevent these infections in individuals at high risk for pneumococcal disease. PCV7, which was the first licensed pneumococcal vaccine to be effective in young children, became available in the beginning of the 21st century and had a tremendous impact on the epidemiology of pneumococci causing disease in children (Vestrheim et al., 2008; Rückinger et al., 2009; Pilishvili et al., 2010; Steens et al., 2013). Remarkably, the use of PCV7 in children protected not only vaccinated children, but also the surrounding non-vaccinated population, with several studies showing that vaccinating children with PCV7 resulted in marked decreases of PCV7-type IPD in adults (e.g. Lexau et al., 2005; Miller et al., 2011b; Harboe et al., 2014). However, at the same time, there were also increases in the incidence of non-PCV7-type IPD in children and adults (Lepoutre et al., 2008; Miller et al., 2011b; Weinberger et al., 2011; Feikin et al., 2013). This situation reduced the benefits of childhood vaccination with PCV7 and led to the development of broader PCVs capable of preventing IPD due to the emerging serotypes. PCV13 replaced PCV7 in the USA and in several countries in Europe. Similarly to PCV7, the use of PCV13 in children was followed by decreases in the incidence of vaccine-type IPD in children and adults (Moore et al., 2015; Tin Tin Htar et al., 2015).

Portugal was for several years in a particular position given that PCVs were used privately in children from June 2001 to June 2015, leading to an uptake below that reached in most other countries adopting PCVs (Aguiar et al., 2008a; Tin Tin Htar et al., 2015; Horácio et al., 2016b). The work presented in this thesis aimed to characterize possible effects of having vaccinated children with PCVs outside the national immunization program on the characteristics of pneumococci causing disease in adults. For that purpose, the serotype and antimicrobial susceptibility of isolates causing adult IPD (Horácio et al., 2013; Horácio et al., 2016b) or adult NIPP (Horácio et al., 2014; Horácio et al., 2018) were determined. For the isolates collected from adult IPD, the clonal structure and prevalence of the pilus islands 1 and 2 were also assessed (Horácio et al., 2016a).

Invasive Pneumococcal Disease

Evolution of Vaccine Serotypes in Adult IPD

The studies of adult IPD presented in this thesis continued an epidemiological surveillance study initiated in 1999. Figure V.S1 shows the evolution of vaccine serotypes in adult IPD in Portugal in the broad period 1999-2014. Previous studies from this epidemiological surveillance network evaluated the possible impact of the private use of PCV7 in children on adult IPD and found that even a low PCV7 uptake in children possibly caused some degree of herd protection in adults against PCV7-type IPD (Aguiar et al., 2008a; Horácio et al., 2012). The proportion of PCV7 serotypes in adult IPD declined from 30.8% in 2004 to 16.6% in 2005 ($p < 0.001$), one year after the first changes occurring in IPD in children that were compatible with the use of PCV7 (Aguiar et al., 2008a). However, even with the significant decline of PCV7 serotypes in adult IPD, PCV7 serotypes still accounted for > 15% of adult IPD in the post-PCV7 period in Portugal (Horácio et al., 2012). These serotypes also persisted as a cause of IPD in children in Portugal (Aguiar et al., 2010a). This remaining burden of PCV7 serotypes in IPD in children and adults in the post-PCV7 period contrasted with the reality in the USA, where PCV7 serotypes declined more markedly (Pilishvili et al., 2010). This difference between Portugal and the USA was probably associated with the slow uptake and lower coverage of PCV7 in children in Portugal.

Another important change accompanying the use of PCVs in children was the significant increase of non-PCV7 type IPD (Lepoutre et al., 2008; Muñoz-Almagro et al., 2008; Pérez-Trallero et al., 2009; Miller et al., 2011b). Previous data from Portugal (Aguiar et al., 2008a; Horácio et al., 2012) found significant increases in the proportion of particular non-PCV7 serotypes in adult IPD in the post-PCV7 period. The serotypes that increased the most in adult IPD in the post-PCV7 period were serotypes 1, 7F and 19A (Aguiar et al., 2008a; Horácio et al., 2012), which are included in PCV13.

When PCV13 replaced PCV7 in children in Portugal it became important to characterize the potential herd protection of this new vaccine regarding the PCV7 serotypes, which are common to both PCV7 and PCV13, and the additional serotypes included in PCV13. These aims were considered in the studies presented in chapter II of this thesis (Horácio et al., 2013; Horácio et al., 2016b).

Regarding PCV7 serotypes, we found an additional decline in the proportion of these serotypes in adult IPD between the periods 2009-2011 and 2012-2014 (Horácio, et al., 2016b) (Figure V.S1). This additional decline of PCV7 serotypes in adult IPD might have been due to a higher effectiveness of PCV13 in comparison with PCV7 in the elimination in children of the serotypes that are common to both vaccines, with this reflecting in stronger herd protection in adults, or perhaps it was simply the result of the continued use of PCVs in children in the country.

Concerning the additional serotypes of PCV13, we found important declines in their proportion in adult IPD in the post-PCV13 period (Figure V.S1) (Horácio et al., 2016b). However, while the decreases of some of the additional PCV13 serotypes were consistent with herd protection caused by childhood vaccination with PCV13, other serotypes started to decrease in adult IPD too soon to represent herd protection due to the use of PCV13 in children (Horácio et al., 2013). This was the case of serotypes 1 and 5 that significantly decreased in adult IPD from 2008 onwards. Given that PCV10 became available for children in Portugal only in mid-2009 and PCV13 only in early-2010, the lower values found for these two serotypes in adult IPD in 2009 and 2010 could not have been associated with childhood vaccination with these two vaccines. In addition, it is known that herd protection takes some time to establish (Aguiar et al., 2008a; Miller et al., 2011a), which means that only data from 2011-2012 onwards would represent with more certainty possible herd protection due the use of PCV13 in children in adult IPD. Serotype 1 is associated with secular trends with long term fluctuations (Fenoll et al., 2009; Harboe et al., 2010) and serotype 5 was found to be responsible for outbreaks (Vanderkooi et al., 2011). Therefore, the significant decreases of serotypes 1 and 5 in adult IPD from 2008 to 2011 may be related with these features (Horácio et al., 2013).

The declines in the PCV13 additional serotypes that we believe were consistent with the use of PCV13 in children in Portugal were those found for serotypes 7F and 19A (Horácio et al., 2016b). This is because these two serotypes declined significantly in adult IPD when a herd effect was expected. In addition, serotypes 7F and 19A also decreased as causes of IPD in children and adults in several other countries following the use of PCV13 in children (Harboe et al., 2014; Moore et al., 2015; Waight et al.,

2015). Moreover, in countries only using PCV10, serotype 19A did not declined in IPD in the post-PCV10 period (Tin Tin Htar et al., 2015).

In studies evaluating proportions and not incidences, such as those presented in this thesis, decreases in the overall proportion of PCV13 serotypes are naturally compensated by increases of the same magnitude in the overall proportion of non-PCV13 serotypes. However, among the two groups of non-PCV13 serotypes considered in our studies – PPV23 additional serotypes and non-vaccine types – it was the group of PPV23 additional serotypes that accounted for most of the increase found from 2008 to 2014 (Figure V.S1) (Horácio et al., 2013; Horácio et al., 2016b). Additionally, among all the usually detected non-PCV13 serotypes in adult IPD, only some serotypes increased significantly. These were, in decreasing frequency, serotypes 8, 22F, 20 and 15A, which also increased in other countries from Europe and North America in a similar period (e.g. Demczuk et al., 2013; Moore et al., 2015; Waight et al., 2015). Importantly, in Portugal, these serotypes increased not only in proportion but also in absolute numbers. This situation was noteworthy at least in the last two years studied (2013 and 2014), because the increases in these four serotypes were opposed to declines in the total number of isolates recovered from adult IPD in each year (Horácio et al., 2016b). Hence, another finding of the studies presented in this thesis (Horácio et al., 2013; Horácio et al., 2016b) was that particular non-PCV13 serotypes emerged as causes of adult IPD in Portugal during the 2008-2014 period.

In conclusion, in the studies presented in chapter II of this thesis (Horácio et al., 2013; Horácio et al., 2016b) we found that, as what happened with PCV7 in Portugal, even a relatively moderate uptake of PCV13 in children likely resulted in some degree of herd protection in adults against PCV13-type IPD. Nevertheless, the increase of non-PCV13 serotypes in adult IPD is concerning. The evolution of these non-PCV13 serotypes should be monitored in future surveillance studies.

Serotype Distribution and Characteristics of the Most Frequent Serotypes Causing Adult IPD

The relative proportion of each serotype causing adult IPD between 2009 and 2014 was detailed in the studies presented in chapter II of this thesis (Horácio et al., 2013; Horácio et al., 2016b). We found a high diversity of serotypes, as demonstrated

by the high SIDs (≥ 0.93) and the ≥ 50 different serotypes identified in each year. However, similarly to what was found before with data collected from 1999 to 2008 (Serrano et al., 2004; Aguiar et al., 2008b; Horácio et al., 2012), only a limited number of serotypes were highly frequent and accounted for the majority of adult IPD.

When considering together all adult IPD isolates recovered from 1999 to 2014, the 10 most frequent serotypes, which accounted for 65.5% of the entire collection of adult IPD isolates (n = 3019 / 4610) were, in decreasing frequency, serotypes 3, 7F, 19A, 14, 1, 8, 22F, 4, 11A and 9N. Figure V.S2 shows the distribution of these 10 serotypes according to different periods of the broad period 1999-2014. Despite yearly fluctuations in the representation of the individual serotypes in adult IPD, together these 10 serotypes accounted for at least 60% of the isolates recovered in each period.

It is known that only a limited number of serotypes cause the majority of IPD globally (WHO, 2008; Johnson et al., 2010) and in agreement with this knowledge, the 10 most frequent serotypes occurring in adult IPD in Portugal in 1999-2014 were also identified as highly prevalent in adult IPD in other countries (Demczuk et al., 2012; Steens et al., 2013). However, there were still important differences between the serotype distribution of adult IPD in Portugal and the serotype distribution of adult IPD in other countries. For example, serotype 12F was a common cause of adult IPD in the USA and in Israel in the post-PCV13 period (Moore et al., 2015; Regev-Yochay et al., 2015), but was rarely found in our collection of adult IPD isolates in a similar period (Horácio et al., 2016b). In addition, even though some serotypes may be equally prevalent between countries, they may still have different clonal structures. One example is the case of serotype 19A that increased in the post-PCV7 period in several countries, including in Portugal, due to the expansion of different genetic lineages (Aguiar et al., 2010b).

The polysaccharide capsule is the major determinant of pneumococcal invasiveness since acapsular isolates are barely found causing invasive disease (Horácio et al., 2016b). However, for some serotypes not all genetic lineages are equally represented in disease (Serrano et al., 2005; Beall et al., 2006; Horácio et al., 2016a). This highlights the importance of other factors, besides the capsule, for the outcome of human-pneumococcal interaction. It is thought it is the combination of the polysaccharide capsule and the array of virulence factors of a given pneumococcal

strain that influences its ability to cause disease. Factors associated with the host immune system also condition the interaction of pneumococci with humans, as shown by the existence of several risk groups for pneumococcal infection (Kyaw et al., 2005; Goldblatt and O'Brien, 2015; Torres et al., 2015). The studies presented in this thesis did not aim to evaluate the reasons underpinning the success of the most frequent serotypes occurring in adult IPD in Portugal, but we still evaluated the correlation of all serotypes with some properties, namely patient's age, antimicrobial susceptibility, presence and type of pneumococcal pilus islands and MLST-defined genotypes (Horácio et al., 2013; Horácio et al., 2016a; Horácio et al., 2016b).

Serotype 3 was the most common serotype causing adult IPD in Portugal in the broad period 1999-2014 (Fig. V.S2). In fact, this serotype was the most frequent serotype in all studied periods included in this broad period, except in 1999-2003, when it occurred immediately after serotype 14. Serotype 3 is targeted by PCV13, but it did not decline in proportion in adult IPD in the post-PCV13 period (Horácio et al., 2016b). By the time we have written the second manuscript of chapter II, the available literature also reported a lack of a consistent reduction of serotype 3 in adult IPD in the post-PCV13 period (e.g. Steens et al., 2013; Harboe et al., 2014; Moore et al., 2015; Waight et al., 2015). However, a later published study from Israel (Regev-Yochay et al., 2017), which analyzed adult IPD isolates after ~ 5 years of PCV13 use in the national immunization program of children, showed a small but consistent decline in the incidence of serotype 3 IPD. In addition, this serotype declined in adult NIPP in the post-PCV13 period in Portugal (Horácio et al., 2018) and elsewhere (Rodrigo et al., 2015). Perhaps a continued use of PCV13 in children is needed before consistent results emerge regarding the ability of PCV13 use in children to eliminate serotype 3 in adult IPD.

Serotype 3 was described in one study to resemble "opportunistic pathogens" in that it caused IPD mostly in patients with underlying disease (Sjöström et al., 2006). Even though in two of our studies (Horácio et al., 2012; Horácio et al., 2013) serotype 3 showed an association with elderly patients, it was still common in the younger age group. This means the debilitated health state of the elderly cannot by itself explain the high prevalence of serotype 3 in adult IPD in Portugal. Instead, specific characteristics of the isolates expressing serotype 3 must also have been important for

its high prevalence among adults of all ages. Serotype 3 was not associated with antimicrobial resistance during the broad period 1999-2014 and so its high frequency as a cause of adult IPD was not related with this feature. Even though the polysaccharide capsule of serotype 3 isolates may have played a role in the success of this serotype in adult IPD, we found that not all genetic lineages expressing serotype 3 were equally prevalent in 2008-2011 (Horácio et al., 2016a). In this period (2008-2011), all except one isolate expressing serotype 3 lacked the two pilus islands, meaning that the presence of pili 1 and 2 did not influence the different prevalence of the distinct genetic lineages associated with serotype 3 in adult IPD. In 2008-2011, CC180 was the dominant clonal cluster among serotype 3 isolates and we found this CC was selected in relation to two other clonal clusters, which were CC260 and CC458, when comparing data from periods 1999-2002 and 2008-2011 (Horácio et al., 2016a). A dominance of CC180 among isolates expressing serotype 3 has also been reported in other countries (Beall et al., 2006; Muñoz-Almagro et al., 2011). Therefore, it is possible that CC180 presents some traits justifying its high prevalence in adult IPD and dominance in relation to other genetic lineages associated with serotype 3. This could be the object of future research.

Serotype 14 was the fourth most frequent serotype causing adult IPD in 1999-2014 (Fig. V.S2). This serotype is targeted by all the PCVs available to date and probably due to indirect protection caused by PCV7 use in children declined in proportion in adult IPD in the post-PCV7 period (Aguiar et al., 2008a). Even though this decline was significant, serotype 14 remained an important cause of IPD in adults in the post-PCV7 period. In fact, the prevalence of serotype 14 remained relatively constant following the decline, with only an additional small decrease in 2014 (Horácio et al., 2016b). Other countries reported stronger decreases of serotype 14 in adult IPD following PCV7 use in children (Miller et al., 2011b; Demczuk et al., 2012), but vaccine coverage was higher in those places. Therefore, the use of PCV13 in the national immunization program of children since June 2015 possibly resulted in a more marked decline of serotype 14 in adult IPD in Portugal from 2015 onwards.

Serotype 14 was strongly associated with antimicrobial resistance throughout the 1999-2014 period (Aguiar et al., 2008a, Horácio et al., 2012; Horácio et al., 2013; Horácio et al., 2016b) and with the presence of pilus island 1 in the 2008-2011 period

(Horácio et al., 2016a). However, the two clonal clusters expressing serotype 14 in 2008-2011, which were CC156 and CC15, were differently correlated with these two characteristics (Horácio et al., 2016a). While all isolates expressing serotype 14 and belonging to CC156 were PNSP (in fact, < 50% of these isolates were EPNSP) and presented PI-1, all serotype 14 isolates from CC15 were ERP and did not present any of the pilus islands. From 2008 to 2009, CC156 expanded within serotype 14, while CC15 reduced. Therefore, CC156 intrinsic properties may have promoted the expansion of CC156 as a cause of adult IPD in 2009. One of the consequences of the growth of CC156 was an overall increase in penicillin-non-susceptibility from 2008 to 2009. However, we found that erythromycin resistance decreased within serotype 14 from 2012 to 2014. The solid association of CC15 with erythromycin resistance in the 2008-2011 period suggests this later change in resistance within serotype 14 was linked to additional shrinking of CC15. Further studies are needed to explore this idea since erythromycin resistance also existed among CC156 isolates expressing serotype 14 (Horácio et al., 2016a).

Serotype 4 was the eighth most frequent serotype causing adult IPD in 1999-2014 (Fig. V.S2) and, as serotype 14, declined significantly in adult IPD following the use of PCV7 in children in Portugal (Aguiar et al., 2008a). Serotype 4 was the second most frequent PCV7 serotype in all studied periods of the broad period 1999-2014, following always serotype 14. The only exception was the 2012-2014 period, when this serotype was also surpassed by PCV7 serotype 19F (Horácio et al., 2016b). Almost all isolates expressing serotype 4 were susceptible to the antibiotics tested, a factor that may have helped in the strong decline of serotype 4 in the post-PCV7 period (Aguiar et al., 2008a; Horácio et al., 2012; Horácio et al., 2013). The most important findings regarding the clonal structure of serotype 4 in 2008-2011 were: its high genetic diversity with no dominant genotype; the association of some genetic lineages expressing serotype 4 with pilus island 1 (e.g. CC205 and CC1221); and the association of one clonal cluster, which was CC5902 (ST1222 and ST801), with the younger age group (Horácio et al., 2016a). Since we did not detect any genotype selection within serotype 4 and because there was no dominant genetic lineage among serotype 4 isolates in the 2008-2011 period it is difficult to suggest a role for the different genetic lineages associated with this serotype on the relatively high prevalence of serotype 4

in adult IPD in Portugal. Perhaps in the case of serotype 4, the polysaccharide capsule was the most important determinant of invasiveness.

Serotype 1, which ranked fifth in the list of the 10 most common serotypes in adult IPD in 1999-2014 (Fig. V.S2), was particularly frequent in adult IPD from 1999 to 2008 (Aguiar et al., 2008a; Horácio et al., 2012). A high prevalence of serotype 1 was also detected in other European countries in a similar period (Fenoll et al., 2009; Harboe et al., 2010). In our studies, serotype 1 was mostly susceptible to the antibiotics, was significantly associated with the younger age groups (Horácio et al., 2013; Horácio et al., 2016b) and presented very low genetic diversity (Horácio et al., 2016a). The dominant clonal cluster was CC306, which was positively associated with pilus island 2 (Horácio et al., 2016a). One study found that isolates from ST306 were highly prevalent as a cause of IPD but rarely carried asymptomatically, suggesting high invasive disease potential for this genetic lineage (Sá-Leão et al., 2011). The high invasiveness of serotype 1 and its preference for the younger adult age groups supports the idea that serotype 1 isolates act as primary pathogens in Portugal. In our country, serotype 1 has been associated with empyema (Aguiar et al., 2010a), attesting also to its virulence. The fact that younger patients usually have less comorbidities than older patients may explain why infections due to isolates of ST306 rarely result in death of the patient (Harvey et al., 2011). Infections due to other genetic lineages associated with serotype 1, such as CC217, were however associated with high mortality in Africa (Harvey et al., 2011). We found that two of our serotype 1 isolates collected from adult IPD in 2011 were of CC217 (Horácio et al., 2016a). This would be a major concern if there were no effective vaccines today for the prevention of serotype 1 infections. Even so, the evolution of serotype 1 isolates in Portugal should be monitored in future studies.

Serotypes 7F and 19A, which were, respectively, the second and third most common serotypes causing adult IPD in 1999-2014 (Fig. V.S2), exhibited similar trends throughout this broad period. In summary, both serotypes expanded in the post-PCV7 period (Aguiar et al., 2008a; Horácio et al., 2012) and declined in the post-PCV13 period (Horácio et al., 2016b). However, the characteristics associated with serotypes 7F and 19A differed in several ways. Serotype 7F isolates, resembling serotype 1 isolates instead, were in our studies highly susceptible to the antibiotics tested (e.g. Horácio

et al., 2013), were associated with pilus island 2 and with only one CC, namely CC191 (Horácio et al., 2016a), which was found in one study to be highly invasive (Sá-Leão et al., 2011). However, in contrast to serotype 1, no association with age was found for serotype 7F in our studies (Horácio et al., 2013; Horácio et al., 2016b). Serotype 19A, on the other hand, shared several traits with serotype 14. In 2008-2011, serotype 19A isolates presented high genetic diversity and the different genetic lineages expressing this serotype were also differently associated with antimicrobial resistance and pili (Horácio et al., 2016a). In addition, our data suggested genotype selection within serotype 19A (Horácio et al., 2016a). The dominant clonal cluster among serotype 19A isolates in 2008-2011, which was CC230, was mostly composed by EPNSP and did not present pilus islands, while the second most frequent clonal cluster among serotype 19A isolates, which was CC199, presented pilus island 1 and was mostly susceptible to the antibiotics tested. From 2008 to 2009, CC230 expanded among serotype 19A isolates despite a decrease in the total number of isolates expressing this serotype. This expansion of CC230 led to an increase in penicillin-non-susceptibility within serotype 19A in this period (Horácio et al., 2016a). However, we also noted that from 2012 to 2014 the proportion of ERP and PNSP decreased within serotype 19A (Horácio et al., 2016b). Therefore, changes in the genetic lineages expressing serotype 19A may have occurred in this period and deserve further evaluation.

Serotypes 8 and 22F, which were, respectively, the sixth and seventh more common serotypes causing adult IPD in the broad period 1999-2014 (Fig. V.S2), significantly increased in adult IPD since the late post-PCV7 period (Horácio et al., 2013; Horácio et al., 2016b). These two serotypes shared some characteristics, such as their association with antimicrobial susceptibility in all studied periods (e.g. Horácio et al., 2013; Horácio et al., 2016b) and the fact that at least in the 2008-2011 period both serotypes presented low genetic diversity and lacked the two pilus islands (Horácio et al., 2016a). However, serotype 8 was found to be associated with the younger age group in 2009-2011 (Horácio et al., 2013), while no association with age was detected for serotype 22F. Additionally, representatives of the dominant clonal cluster among serotype 8 isolates, which was CC62 (ST53), were found in one study to be highly invasive, while representatives of the most important clonal cluster among serotype 22F isolates, which was CC433, were found to be equally prevalent in IPD and carriage

(Sá-Leão et al., 2011). These last two characteristics of serotype 8 were shared with serotype 1 instead, as discussed above. The increase of serotype 8 as a cause of adult IPD in the post-PCV7 period was more notorious than that of serotype 22F, and in 2013 and 2014, serotype 8 became the second most common cause of adult IPD in Portugal (Horácio et al., 2016b). Importantly, of these two serotypes, only serotype 22F is covered by the 15-valent PCV that is under evaluation (Skinner et al., 2011). The strong increase of serotype 8 in adult IPD in the post-PCV7 and post-PCV13 period stresses the need to continue following IPD due to this serotype.

Serotypes 9N and 11A, which were, respectively, the ninth and tenth most frequent serotypes causing adult IPD in 1999-2014 (Fig. V.S2), were consistently recovered from adult IPD throughout the entire study period (1999-2014). These two serotypes shared some characteristics, such as antimicrobial susceptibility and the absence of pilus islands. Serotype 9N presented very low genetic diversity and had all but one isolate included in the same clonal cluster, which was CC81. Similarly, and despite the higher genetic diversity of serotype 11A, all except one isolate expressing serotype 11A were included in only one clonal cluster, which was CC62 (Horácio et al., 2013; Horácio et al., 2016a; Horácio et al., 2016b).

In summary, only a limited number of serotypes caused the majority of adult IPD in Portugal during the broad period 1999-2014, suggesting that these serotypes may have some properties contributing to their high frequency in adult IPD. The 10 most common serotypes in adult IPD in 1999-2014 associated differently with the three age groups considered, with antimicrobial resistance and with pili, and while some of these serotypes presented a complex non-static clonal structure, others presented low genetic diversity. The reasons behind the high frequency of these 10 serotypes as a cause of adult IPD in Portugal were therefore possibly multifactorial and this could be the object of future research.

Overall Genetic Diversity in Adult IPD

The study of MLST presented in chapter III of this thesis (Horácio et al., 2016a) was important to better understand the diversity of isolates occurring within each serotype and to evaluate the stability of the genetic lineages. We opted to determine the genetic lineages of all serotypes causing adult IPD in 2008-2011 (i.e. 52 different

serotypes), as this would give us a clearer view of the pneumococcal population causing adult IPD in Portugal in this period and how it adapted during the time of private PCVs use in children. The period chosen for analysis corresponds to the last two years of PCV7 private use in children in Portugal (2008 and 2009) and the first two years of PCV13 private use in children in the country (2010 and 2011). These results were compared with previously published data from the same network (Serrano et al., 2005).

In summary, we found the isolates responsible for adult IPD in 2008-2011 presented high genetic diversity (SID = 0.971, CI95%: 0.967–0.976), with 206 different STs being detected among the sample of 871 isolates (Horácio et al., 2016a). These STs distributed into 80 different clonal clusters, according to goeBURST analysis (Francisco et al., 2009). Despite this high diversity of STs and CCs, only 14 STs and 6 CCs accounted for half of the isolates analysed. The more frequent clonal clusters were mainly composed of isolates expressing vaccine serotypes, which was not surprising due to the high frequency of these serotypes in 2008-2011. The major clonal cluster was CC156, which was mostly composed of isolates expressing PCV7 serotypes (namely serotypes 14, 9V and 23F). Several of the genetic lineages found to be causing IPD in adults in Portugal were also detected as causes of IPD in other countries (e.g. Muñoz-Almagro, et al., 2011; Pichon et al., 2013; Caierão et al., 2014; Metcalf et al., 2016).

As already discussed, there were important changes in the serotype distribution of adult IPD isolates during the time of PCVs use in children, including in the 2008-2011 period (Horácio et al., 2012; Horácio et al., 2013). We found the changes in the serotype distribution occurring in adult IPD in Portugal in this period were accompanied by decreases and expansions in the proportions of the associated genetic lineages. The most important of these changes was the significant decline of CC306, which accompanied the decline of its associated serotype (serotype 1), from 2008 onwards (Horácio et al., 2013).

Another goal of the study presented in chapter III of this thesis (Horácio et al., 2016a) was to evaluate the importance of capsular switching among the isolates collected from adult IPD. Capsular switching is known to occur in pneumococci and a concern exists that successful lineages expressing vaccine serotypes may change

their capsules to non-vaccine serotypes and escape vaccine control, as reported before (Brueggemann et al., 2007). Increases of non-vaccine serotypes after the availability of PCVs may be associated with these events and therefore, need to be evaluated. Our data suggested capsular switching was rare among the isolates causing adult IPD in 2008-2011. Worth of attention was only a case of a possible capsular switching from the PCV13 serotype 19A to the NVT 24F. We saw that in the pre-PCV7 period, serotype 24F was mainly CC81, while in the 2008-2011 period among the nine 24F isolates genotyped, four were CC81 and five were CC230. This finding is relevant because ST276, which is an SLV of ST230, led to the expansion of serotype 19A in the post-PCV7 period in Portugal and this genetic lineage is associated with antimicrobial resistance (Aguiar et al., 2010b). This combination was also detected in other countries (Pantosti et al., 2002; Simões et al., 2011) and hence, close attention should be paid to its evolution in the future.

In what concerns the overall prevalence of pilus islands 1 and 2 among the isolates causing adult IPD in 2008-2011, we found that almost 1/3 of the isolates (32%) presented a pilus island (Horácio et al., 2016a). As expected (Aguiar et al., 2008b), there was an association between the presence and type of pilus islands with serotype, and an even stronger association between the presence and type of pilus islands with genotype. PI-1 was mainly found among isolates expressing PCV7 serotypes, while PI-2 among isolates expressing the additional serotypes covered by PCV13. The additional serotypes of PCV13 were common causes of adult IPD in 2008-2011 and this explains the higher frequency of PI-2 positive isolates (19%) over PI-1 positive isolates (12%), found in this pneumococcal collection. The decrease in proportion of serotype 1 in adult IPD in 2008-2011 led to a significant decline in proportion of invasive isolates bearing PI-2 (from 25% in 2008 to 16% in 2011, $p = 0.007$). Contrasting with results from one study showing the re-emergence of pneumococcal isolates bearing PI-1 in a population of children in Massachusetts (Regev-Yochay et al., 2010), our data suggests that in Portugal there was no re-emergence of PI-1 in pneumococci causing adult IPD after the continued use of PCVs in children. The overall prevalence of the PIs in adult IPD may be even smaller in 2012-2014, due to the significant decline of serotype 7F in the post-PCV13 period (Horácio et al., 2016b), which was also associated with PI-2 (Horácio et al., 2016a).

Overall Antimicrobial Susceptibility in Adult IPD

Since five of the seven serotypes targeted by PCV7 have been associated with antimicrobial resistance (Dagan and Klugman, 2008), the use of PCV7 in children was expected to reduce invasive disease caused by antimicrobial resistant pneumococci. However, the emergence of the resistant serotype 19A in the post-PCV7 period prevented this from happening in some countries, especially in adults (Horácio et al., 2012; Pérez-Trallero et al., 2009). PCV13 targets serotype 19A and since no serotype-like 19A seems to have emerged in the post-PCV13 period in countries using PCV13 (Tin Tin Htar et al., 2015), a decrease in the representation of antimicrobial resistant isolates in IPD can, once again, be expected.

In the studies of chapter II of this thesis (Horácio et al., 2013; Horácio et al., 2016b), all isolates collected from adult IPD from 2009 to 2014 were tested for susceptibility to several classes of antibiotics, continuing the epidemiological surveillance initiated in 1999 (Serrano et al., 2004; Aguiar et al., 2008a; Horácio et al., 2012). The classes of antibiotics used for testing the isolates were chosen according to their clinical or epidemiological relevance.

When considering the broad period 1999-2014 (Serrano et al., 2004; Aguiar et al., 2008a; Horácio et al., 2012; Horácio et al., 2013; Horácio et al., 2016b), all adult IPD isolates were found to be susceptible to vancomycin and linezolid. Resistance to levofloxacin was extremely rare and the great majority of isolates were susceptible to cefotaxime and chloramphenicol. For the remaining antibiotics tested, resistance was higher and varied in time.

β-lactams and macrolides have been widely used in the empirical treatment of pneumonia globally (Goldblatt and O'Brien, 2015). The proportions of penicillin-non susceptible pneumococci and erythromycin resistant pneumococci occurring in adult IPD in Portugal in the broad period 1999-2014, and according to the CLSI breakpoints prior to 2008, are represented in Figure V.S3. Previous studies from Portugal reported an increase in the proportion of ERP in adult IPD in the time of PCV7 use in children (i.e., from 1999-2003 to 2008, ERP increased in adult IPD from 10% to 17%) (Aguiar et al., 2008b; Horácio et al., 2012). We found there were new increases in the proportion of ERP in adult IPD until 2010, with this resulting in almost ¼ of adult IPD isolates

being ERP in 2010 (Horácio et al., 2013). Concerning the proportion of PNSP in adult IPD, no significant change was found from 1999 to 2008 in previous studies (Aguiar et al., 2008; Horácio et al., 2012). However, we found there was an increase of PNSP in adult IPD from 2008 to 2010 (from 16% to 24%). The trends found from 1999 to 2010 for ERP and PNSP in adult IPD in Portugal were not only associated with the emergence of serotype 19A in this period, but also with the persistence of PCV7 serotypes in adult IPD, as discussed above (Aguiar et al., 2008; Horácio et al., 2012).

When adding data from 2012 to 2014 (Horácio et al., 2016b), we found that in this period, both the representation of PNSP and ERP declined in adult IPD, though not continuously for ERP (Figure V.S3). These declines of PNSP and ERP in adult IPD were in part promoted by decreases in the number of isolates expressing serotypes 14 and 19A in this period (Horácio et al., 2016b) and in part promoted by decreases of PNSP and ERP even within serotypes 14 and 19A (Horácio et al., 2016b). These decreases were unexpected, but positive. Among the emerging serotypes in the post-PCV13 period, only one serotype was associated with antimicrobial resistance – serotype 15A. The increase of this serotype in adult IPD was much moderate than that noticed before for serotype 19A in the post-PCV7 period (Aguiar et al., 2008a), which agrees with the idea that no serotype-like 19A have emerged in the post-PCV13 period (Tin Tin Htar et al., 2015). Given that serotypes 14 and 19A still contributed to antimicrobial resistance in the last year of the study (i.e. 2014), the inclusion of PCV13 in the national immunization program of children, since June 2015, is expected to further reduce the prevalence of resistant isolates in adult IPD.

In the studies presented in chapter II of this thesis (Horácio et al., 2013; Horácio et al., 2016b) we decided to use the CLSI breakpoints for penicillin prior to 2008 throughout the entire study period (1999-2014) to better understand the changes occurring in pneumococci causing invasive disease in adults. However, we also reported the results considering the current cut-off values (Clinical and Laboratory Standards Institute, 2015). According to the current breakpoints, the great majority of isolates recovered from non-meningitis cases in 2009-2014 were classified as susceptible to penicillin (0.5% of resistance in 2012-2014) (Horácio et al., 2013; Horácio et al., 2016b). This result agrees with international and national guidelines suggesting

the use of β-lactams for the empirical treatment of pneumonia (Direção Geral da Saúde, 2011; Goldblatt and O'Brien, 2015).

Non-invasive Pneumococcal Pneumonia

Evolution of Vaccine Serotypes in Adult NIPP

Non-invasive pneumococcal pneumonia is estimated to be much more frequent than invasive pneumococcal pneumonia (Said et al., 2013) and to account for a significant fraction of pneumococcal associated deaths (Cillóniz and Torres, 2012). However, several factors, such as the challenges associated with the etiological diagnosis of NIPP (Goldblatt and O'Brien, 2015), complicate appropriate epidemiological surveillance of these infections, with this situation reflecting in a limited number of NIPP studies. The last two studies presented in this thesis (Horácio et al., 2014; Horácio et al., 2018) were dedicated to the analysis of pneumococci causing adult NIPP in Portugal. Similarly to what we have done in the studies of adult IPD (Horácio et al., 2013; Horácio et al., 2016b), we aimed to evaluate the characteristics of pneumococci causing non-invasive pneumococcal pneumonia in adults in Portugal and how these characteristics changed in time with the private use of PCVs in children. For that purpose, we analysed isolates collected from adult NIPP between 1999 and 2015. We also compared adult NIPP data with adult IPD data to evaluate if adult IPD data could be used to extrapolate information of adult NIPP, as suggested elsewhere (van Werhoven et al., 2016).

We found that childhood private vaccination with PCVs in Portugal possibly caused herd effects not only on pneumococci responsible for adult IPD, but also on pneumococci responsible for adult NIPP. However, the overall proportion of vaccine serotypes was usually lower in adult NIPP than in adult IPD, and the serotypes that changed the most in the post-PCVs period in adult NIPP did not match entirely with those that changed the most in adult IPD in a similar period. In addition, adult NIPP and adult IPD presented different serotype distributions, with several serotypes being associated with either disease presentation (Horácio et al., 2014; Horácio et al., 2018).

There were changes in the serotype distribution in adult NIPP in the 1999-2015 period that suggested herd protection in adults due to the use of PCVs in children.

Figure V.S4 shows the evolution of vaccine serotypes in adult NIPP in the broad period 1999-2015. Concerning the representation of PCV7 serotypes in adult NIPP, the proportion of these serotypes declined gradually in adult NIPP in the post-PCV7 period (Horácio et al., 2014) and then remained slightly above 10% from 2008 to 2015 (Horácio et al., 2018). This suggests that PCV7 and PCV13 use in children in Portugal protected adults from PCV7-type NIPP. However, and as what happened in adult IPD, PCV7 serotypes did not disappear as causes of adult NIPP in Portugal (Figure V.S4). As discussed above for adult IPD, this situation may be the result of the use of PCVs outside the national immunization program of children until June 2015.

Regarding the additional serotypes of PCV13, we found a significant decline in their proportion after the availability of PCV13 for private use in children (declined from 36% in 2010 to 19% in 2015, $p < 0.001$; Figure V.S4) (Horácio et al., 2018). The PCV13 serotypes that decrease the most were serotypes 3 and 19A. The decrease of serotype 3 in adult NIPP was surprising since this serotype did not show a consistent decline in adult IPD in Portugal (Horácio et al., 2016b) nor in other countries (e.g. Steens et al., 2013; Harboe et al., 2014; Moore et al., 2015; Waight et al., 2015) in the early post-PCV13 period, as discussed above. However, a decrease in the incidence of NIPP associated with serotype 3 was reported elsewhere (Rodrigo et al., 2015). Perhaps a decline of serotype 3 in adult IPD in Portugal will occur now that PCV13 is implemented in the national immunization program of children. In what concerns the decline of serotype 19A in adult NIPP it was much less pronounced than that registered for adult IPD in the post-PCV13 period (Horácio et al., 2018). Due to the strong evidence of herd protection resulting from childhood vaccination against serotype 19A in adult IPD (Tin Tin Htar et al., 2015), it is possible that the universal vaccination of children with PCV13, now implemented in Portugal, may also cause a stronger decrease of serotype 19A in adult NIPP. The evolution of serotypes 3 and 19A as a cause of disease in adults in Portugal should be monitored.

As discussed above for adult IPD, in studies evaluating proportions and not incidences, such as those presented in this thesis, declines in the global proportion of PCV13 serotypes are consequently compensated by increases in the remaining groups of serotypes. However, among the two other groups considered in our studies (i.e. PPV23 additional serotype and NVTs), only NVTs increased significantly in adult

NIPP in the post-PCV13 period (Figure V.S4). This contrasted with results from adult IPD, for which the decrease in the global proportion of PCV13 serotypes was accompanied by an increase in the overall proportion of PPV23 additional serotypes (Horácio et al., 2016b). While the overall increase of PPV23 additional serotypes in adult IPD was mainly promoted by important increases of particular serotypes, with this suggesting the existence of emerging serotypes in adult IPD, the same did not happen in adult NIPP, since none of the NVTs showed a remarkable increase in adult NIPP in the post-PCV13 period (Horácio et al., 2018).

In conclusion, in the studies presented in chapter IV of this thesis (Horácio et al., 2014; Horácio et al., 2008) we found that even a relatively moderate uptake of PCV7 and PCV13 in children possibly caused herd protection in adults against vaccine-type NIPP. However, PCV13 serotypes persisted as causes of adult NIPP following the private use of PCV13 in the country, highlighting the importance of the implementation of PCV13 in the national immunization program of children to reduce the circulation of these serotypes. Fortunately, none of the serotypes not covered by PCV13 seemed to have emerged in adult NIPP in the post-PCV13 period. Since the evolution of vaccine serotypes in adult NIPP in the post-PCVs period differed from that reported for adult IPD, studies focusing specifically on adult NIPP are also needed.

Serotype Distribution in Adult NIPP

The relative proportion of each serotype causing adult NIPP in 1999-2015 was detailed in the studies presented in chapter IV of this thesis (Horácio et al., 2014; Horácio et al., 2018). As what was found for adult IPD, there was high diversity of serotypes among the isolates collected from adult NIPP in Portugal. In the first study period (1999-2011), 57 different serotypes were detected as causes of adult NIPP and SID values ranged from 0.901 to 0.957 in each year, while in the second study period (2012-2015), 50 different serotypes were detected as causes of adult NIPP and SID values ranged from 0.944 to 0.955 in each year. Despite the high diversity of serotypes, some serotypes were more frequently detected than others. The 10 most common serotypes causing adult NIPP when considering the whole study period together (1999-2015) were, in decreasing frequency, serotypes 3, 11A, 19F, 19A, 22F, 6C, 9N, 23A,

14 and 23B. Their proportions, according to the different periods within the broad period 1999-2015, are represented in Figure V.S5. These 10 serotypes were found in 54.7% of the adult NIPP collection (n = 1497 / 2735), a value that is much lower than that found above for adult IPD (65.5%), when performing a similar analysis. The next more frequent serotypes in adult NIPP in the broad period 1999-2015 were serotypes 15A, 15B/C, 31 and NTs, with each accounting for 2.3% to 2.8% of the 2735 isolates.

Among the 10 most frequent serotypes causing adult NIPP in 1999-2015 (Figure V.S5), six serotypes were also amongst the 10 most frequent serotypes causing adult IPD during the period 1999-2014 (Figure V.S2), namely serotypes 3, 11A, 19A, 22F, 9N and 14. However, when comparing similar periods for adult IPD and adult NIPP, we found serotype 11A to be significantly more represented in adult NIPP than in adult IPD and serotypes 19A, 22F and 14 to be significantly more represented in adult IPD than in adult NIPP (Horácio et al., 2014; Horácio et al., 2018). In addition, several other serotypes from the list of the most frequent serotypes in adult NIPP and adult IPD showed an association with one or the other disease presentation (i.e. serotypes 19F, 23A and 23B with adult NIPP and serotypes 7F, 1, 8 and 4 with adult IPD) (Horácio et al., 2014; Horácio et al., 2018). Serotypes 11A, 19F, 23A and 23B were found to be associated with carriage instead of IPD in a study evaluating the invasiveness of the genetic lineages circulating in Portugal (Sá-Leão et al., 2011). The suggestion of a low invasiveness for these four serotypes (Sá-Leão et al., 2011) agrees with their association with adult NIPP instead of adult IPD found in our studies.

The different serotype distributions of adult NIPP and adult IPD resulted in a different representation of the serotypes included in PCVs in the two collection of isolates. For example, for both adult IPD and adult NIPP the highest representation of PCV13 serotypes occurred in 2008, but while the value reached for adult IPD was 70%, for adult NIPP it was only 57% (Figures V.S2 and V.S5). We did not evaluate the genetic lineages of the isolates responsible for adult NIPP in Portugal for any of the studied periods, but the different serotype distributions found between adult NIPP and adult IPD suggests their genetic lineages may also not match entirely if such analysis is performed. For the future it will be important to characterize the population structure of pneumococci responsible for adult NIPP, since this may give a clear view of the differences between pneumococci responsible for adult NIPP and

adult IPD. In addition, it may also contribute to understand the reasons behind the high frequency of particular serotypes in adult NIPP.

Since we did not determine the population structure, nor the distribution of pili, for the isolates collected from adult NIPP and because no significant associations were found between the different serotypes detected in adult NIPP with the three age groups considered in the studies presented in this thesis, it is difficult to discuss on the relative importance of the pneumococcal capsule versus other pneumococcal features for the high representation of these 10 serotypes in adult NIPP, as was done above for adult IPD.

One important finding of the studies presented in chapter IV (Horácio et al., 2014; Horácio et al., 2018) was the realization that serotype 3 was not only the most common cause of adult IPD (Horácio et al., 2013; Horácio et al., 2016b), but also of adult NIPP in Portugal in the time of childhood vaccination with PCVs through the private market. Serotype 3 was also reported has highly prevalent in NIPP in other regions (Domenech et al., 2011; Benfield et al., 2013; Mendes et al., 2015), stressing out its international significance. It is noteworthy that despite the significant decline from 2007 to 2015 in the proportion of serotype 3 in adult NIPP in Portugal, this serotype was still the most frequent serotype in the last years studied (i.e. 2014 and 2015). The importance of serotype 3 as a cause of disease in adults and the fact that isolates expressing this serotype have been found to be genetically diverse, with distinct genetic lineages differently associated with carriage or invasive disease (Sá-Leão et al., 2011), makes this serotype an appealing serotype to study by WGS (Croucher et al., 2013). WGS may also help to identify genetic factors involved in the development of non-invasive pneumonia or invasive disease, and this may be an interesting line of investigation for the future.

Overall Antimicrobial Susceptibility in Adult NIPP

All adult NIPP isolates included in the studies presented in chapter IV of this thesis (Horácio et al., 2014; Horácio et al., 2018) were tested for several classes of antibiotics, similarly to what was performed for adult IPD isolates (Horácio et al., 2013; Horácio et al., 2016b). As also found for adult IPD, all adult NIPP isolates were

susceptible to vancomycin and linezolid. Non-susceptible isolates to levofloxacin, cefotaxime and chloramphenicol were also rare in adult NIPP.

The proportions of PNSP and ERP in adult NIPP in the broad period 1999-2015 and using the 2007 CLSI criteria are shown in Figure V.S6 (Clinical and Laboratory Standards Institute, 2007). In general, antimicrobial resistance among adult NIPP isolates were not that different from that reported for adult IPD in the post-PCV7 period and the use of PCV7 in children also did not reflect in a decrease in the proportion of PNSP or ERP in adult NIPP in the post-PCV7 period. Concerning the evolution of the proportion of PNSP in adult NIPP in the post-PCV7 period, no significant trend was observed over the years, only non-significant fluctuations throughout the entire study period. In contrast, the proportion of ERP increased in adult NIPP in the post-PCV7 period (from 12.4% in 1999-2003 to 27% in 2009). The emergence of serotype 19A and the persistence of PCV7 serotypes in adult NIPP contributed to the results found (Horácio et al., 2014), as what happened for adult IPD (Horácio et al., 2013).

However, while in the post-PCV13 period there was a decline of PNSP and ERP in adult IPD (Horácio et al., 2016b), the same was not replicated in adult NIPP (Horácio et al., 2018). This was mainly due to the higher representation of serotype 19F and NTs in adult NIPP, which are associated with antimicrobial resistance, and to the lower decline of serotype 19A in adult NIPP.

Using current CLSI breakpoints (Clinical and Laboratory Standards Institute, 2015), penicillin non-susceptibility was rare among the isolates causing adult NIPP (1% in 2012-2015). Therefore, not only our adult IPD data but also our adult NIPP data agree with the empirical treatment of pneumonias recommended in Portugal (Direção Geral da Saúde, 2011).

One of the advantages of the etiological diagnosis of pneumonia by bacterial culture over urine antigen detection assays is the fact that the first enables to determine antimicrobial susceptibility profiles of pneumococci. Our studies suggest that not even a different serotype distribution between adult NIPP and adult IPD can reveal if antimicrobial resistance between adult NIPP and adult IPD will also differ.

In conclusion, only in adult IPD were there decreases in the representation of antimicrobial resistant isolates in the post-PCV13 period. The increasing importance

of NTs in adult NIPP in Portugal, which are associated with antimicrobial resistance and which cannot be covered by pneumococcal vaccines based only on polysaccharides, is of concern, and should be followed.

Concluding Remarks and Future Perspectives

In 2015, a total of 6126 people died with pneumonia (ICD-10: J12-J18) in Portugal, with most of these deaths (n = 5840) occurring in adults ≥ 65 yrs (INE, 2015). For unknown reasons, mortality rates due to pneumonia are more pronounced in Portugal than in many other countries from Europe. For example, in 2013, Portugal occupied the second highest position among 23 European countries in a study evaluating mortality rates due to pneumonia (Direção-Geral da Saúde, 2015d). Since *S. pneumoniae* is frequently reported as the most common cause of bacterial pneumonia (e.g. Torres et al., 2014; GBD 2015 Mortality and Causes of Death Collaborators, 2016), one can presume that a significant fraction of pneumonia-associated deaths in Portugal is due to this microorganism. Despite this uncertainty, the large number of pneumococcal isolates recovered from adults with invasive disease or non-invasive pneumonia in each year by this epidemiological surveillance network (e.g. Horácio et al., 2016b; Horácio et al., 2018), highlights the importance of *S. pneumoniae* as a pathogen of adults in Portugal.

The current burden of pneumococcal infections together with the present need for epidemiological studies evaluating the possible herd effects of the use of PCVs in children on adults motivated the studies presented in this thesis. In summary, our data suggested that even a relatively moderate uptake of PCV13 in children possibly changed the serotype distribution of the pneumococcal population responsible for IPD in adults (Horácio et al., 2016b), as what was seen before with PCV7 in Portugal (Aguiar et al., 2008). There were also major changes in the serotype distribution of the pneumococcal population responsible for adult NIPP during the time of PCVs use in children, with some of these changes possibly reflecting herd protection promoted by childhood vaccination with PCVs (Horácio et al., 2014; Horácio et al., 2018).

The wide effect of PCVs in preventing pneumococcal disease in non-vaccinated adults, may question the need of universal vaccination of adults ≥ 65 yrs with PCV13,

which has been ongoing in some countries since age-based recommendations for adults were made (Castiglia et al., 2014; Tomczyk, et al., 2014; Sings et al., 2017). In Portugal, the national health authority recommends the sequential vaccination with PCV13 and PPV23 of adults having increased risk for IPD, but no recommendations have been made to vaccinate adults more broadly, such as all immunocompetent adults ≥ 65 yrs (Direção-Geral da Saúde, 2015b). Several other countries also have risk-based recommendations (Castiglia et al., 2014; Sings et al., 2017). The differences between countries concerning pneumococcal vaccination policies in adults show this is a debatable subject.

If possible, vaccine policies should be based on cost-effectiveness analysis and up to date predictions using the most recent data. According to the last studied years in this thesis (Horácio et al., 2016b; Horácio et al., 2018), 38% of adult IPD in 2014 and 30% of adult NIPP in 2015 were due to PCV13 serotypes. In addition, PPV23 serotypes were expressed by 75% of adult IPD isolates collected in 2014 and 56% of adult NIPP isolates collected in 2015 (Horácio et al., 2016b; Horácio et al., 2018). These proportions are relevant and suggest a potential role of vaccination for the prevention of pneumococcal disease in adults. Since vaccine serotypes were more represented in adult IPD that in adult NIPP throughout the entire post-PCVs period (Horácio et al., 2014; Horácio et al., 2018), and because both PCV13 and PPV23 are more able to prevent adult IPD than adult NIPP (Ochoa-Gondar et al., 2014; Bonten et al., 2015), vaccination of adults in Portugal would have a role especially in the prevention of adult IPD. However, and as stated above, the potential coverage of pneumococcal vaccines in adults likely decreased after 2015. In addition, no cost-effectiveness study regarding the use of pneumococcal vaccines in adults in Portugal is available. In other countries, studies evaluating the cost-effectiveness of adult PCV13 vaccination reached different conclusions (Dirmesropian et al., 2015; van Hoek and Miller, 2016). This situation might represent real differences between countries or might have been due to differences is study design and in the different evidence used as backup in each study (Dirmesropian et al., 2015; van Hoek and Miller, 2016).

Despite all the reports showing that the incidence of PCV13-type IPD in adults declined following the use of PCV13 in children (e.g. Moore et al., 2015; Waight et al., 2015), a steady state in the incidence of adult IPD is yet to be announced in the post-

PCV13 period, not only regarding the serotypes included in PCV13, but also concerning those not covered by this vaccine. Increases in the incidence of non-PCV13-type IPD were noticed in several countries post-PCV13 introduction (e.g. Waight et al., 2015; Regev-Yochay et al., 2017) and it is currently uncertain if these continued thereafter, and if so, to what extent. Given that some non-PCV13 serotypes have been shown to have high invasive disease potential (Sá-Leão et al., 2011), the possibility of serotype replacement in disease after continued use of PCV13, as what happened in some countries following PCV7 use (Lepoutre et al., 2008; Miller et al., 2011b), cannot be ignored. In England and Wales, the increase in non-PCV13 type IPD in children < 5 yrs in 2013-2014 period resulted in a higher overall incidence of invasive pneumococcal disease in this age group, compared with 2012-2013 period (Waight et al., 2015). Our data showed there were significant increases of some non-PCV13 serotypes in adult IPD since the late post-PCV7 period, suggesting emerging serotypes in disease. However, we also noted a significant decrease in the number of invasive isolates recovered from younger adults relative to each of the other two adult age groups considered when comparing the periods 2009-2011 and 2012-2014, with this suggesting a reduction in incidence of IPD in adults 18-49 yrs (Horácio et al., 2016b).

Despite the lack of age-based recommendations from the national health authority, two pneumococcal vaccines are available for use in immunocompetent adults in Portugal, the PPV23 since 1996, and the PCV13 since 2012 (Horácio et al., 2012). In addition, the sequential vaccination of adults ≥ 65 yrs with PCV13 and PPV23 was recommended by two Portuguese medical societies (*Sociedade Portuguesa de Pneumologia* and *Sociedade Portuguesa de Medicina Geral e Familiar*). The lack of wider recommendations from the national health authority in Portugal results in the autonomy of physicians to decide whether to vaccinate immunocompetent adults. In our studies information on the uptake of these vaccines in adults was not available and therefore we could not conclude on the role of adult pneumococcal vaccination in the PCVs era. However, given that indications for the use of PCV13 in adults became available only in late-2011 in Europe, and because studies reporting on the use of PPV23 in adults in the post-PCV7 period in Portugal found it to be very low (Sousa et al., 2009; Fedson et al., 2011), it is unlikely that pneumococcal vaccination of adults in

Portugal have had a significant effect in the results presented in the studies composing this thesis.

The possible impact of PCV10 was also mostly disregarded in the studies presented in this thesis. Even though this vaccine was available for private use in children from mid-2009 to June 2015, PCV13 was the most frequently used pneumococcal vaccine in this period (Horácio et al., 2016b). Given that the use of PCV10 in children is also associated with the induction of herd effects in adults (Tin Tin Htar et al., 2015), there is a possibility that the low use of this vaccine in children in Portugal contributed to the results obtained in the studies presented in this thesis.

In spite of the uncertainties concerning the possible contribution of adult pneumococcal vaccination and childhood PCV10 vaccination for the results obtained in the studies presented in this thesis, we know that other factors besides vaccination were important. The relevance of these other factors became clear, for example, with the significant reductions of serotypes 1 and 5 in adult IPD, which are serotypes targeted by PCV10 and PCV13, immediately before indirect effects of these vaccines could have been possible in Portugal (Horacio et al., 2013). If the reductions of serotypes 1 and 5 in adult IPD had been detected few years later, we could have thought they reflected herd protection with the use of the new vaccines in children. This highlights the importance of carefully evaluating changes in the serotype distribution of pneumococci following the use of PCVs.

Using the current CLSI guidelines (Clinical and Laboratory Standards Institute, 2015), the great majority of pneumococcal isolates collected from non-meningitis cases was susceptible to penicillin (Horácio et al., 2016b; Horácio et al., 2018). As stated before, our results agree with the empirical antimicrobial treatment recommended for pneumonia in Portugal. However, the still high number of deaths associated with pneumonia in Portugal (INE, 2015) and in other countries (GBD 2015 Mortality and Causes of Death Collaborators, 2016), is of concern. To prevent pneumococcal associated deaths, not only effective antimicrobial treatment must be available, but antimicrobial therapy should start as soon as possible (Cillóniz and Torres, 2012). In addition, antibiotics should reach the people in need, which is still an issue in several countries (http://www.who.int/news-room/fact-sheets/detail/pneumonia).

Epidemiological surveillance of pneumococcal disease is important in countries using pneumococcal vaccines, in those preparing to adopt pneumococcal vaccines and in those with high burden of pneumococcal disease. Even though pneumococcal populations may be more similar between neighboring countries than between those far away, and it may be useful to use data from one country to represent countries around, especially in the context of low income countries, several factors may contribute to widen disparities regarding the characteristics of pneumococcal populations causing disease in each country. Examples of such factors are differences in pneumococcal vaccine policies, use of antibiotics and migratory flows. Portugal and Spain are two neighboring countries presenting significant differences regarding the pneumococcal populations causing disease in adults. For example, when comparing post-PCV13 adult IPD data from Portugal (Horácio et al., 2016b) with adult IPD data from Spain in a similar period (Càmara et al., 2017), serotype 8, 22F, 20 and 15A increased significantly in Portugal, while in Spain serotype 8 decreased significantly, serotype 22F increased slightly and no important changes were detected for serotypes 20 and 15A. In addition, serotype 6C increased significantly in Spain, but the same was not reproduced in Portugal. These findings underscore the need to set up epidemiological surveillance networks of pneumococcal disease in all geographic regions, if possible.

Several questions emerged from the results obtain in the studies presented in this thesis and deserve further study: 1) Our data showed there were important changes in the serotype distribution of pneumococci causing adult IPD and adult NIPP, with decreases of PCV13 serotypes in both adult IPD and adult NIPP and increases of specific non-PCV13 serotypes only in adult IPD (Horácio et al., 2016b; Horácio et al., 2018). Therefore, it is important to evaluate how these trends continued in most recent years, if there is evidence of a steady state in the serotype distribution and what is the remaining proportion of disease potentially preventable by the available pneumococcal vaccines. 2) PCV7 serotypes persisted as causes of disease in adults throughout the entire period of private PCVs use in children (Horácio et al., 2016b; Horácio et al., 2018). It would be interesting to see if these serotypes disappeared as causes of adult IPD and adult NIPP with the introduction of PCV13 in the national immunization program of children. 3) There is a believe that with the

continued use of PCV13, PCV7 serotypes will become more represented in disease than the additional serotypes included in PCV13, due to the higher circulation of PCV7 serotypes before introduction of PCV7 (van Hoek and Miller, 2016). It would be interesting to evaluate this hypothesis, since the effectiveness of PCV13 against serotype 3 is currently not well understood (Steens et al., 2013; Harboe et al., 2014; Moore et al., 2015; Waight et al., 2015; Horácio et al., 2016b). 4) If PCV13 use in children can reduce serotype 3, we expect this serotype to no longer be the most prevalent serotype in adult IPD and adult NIPP (Horácio et al., 2016b; Horácio et al., 2018). It is important to evaluate this possibility. In addition, even though serotype 19A showed an important decrease in adult IPD (Horácio et al., 2016b), its decrease in adult NIPP was less relevant (Horácio et al., 2018). The evolution of this serotype as a cause of adult NIPP should also be followed. 5) The most important genetic lineage among serotype 3 isolates from adult IPD was CC180, which we found to have increased within this serotype in the post-PCV13 period (Horácio et al., 2016a). This lineage also seemed to have emerged within serotype 3 causing adult IPD elsewhere (Càmara, et al., 2017). Given the importance of serotype 3 as a cause of disease in adults in Portugal, it would be interesting to evaluate why CC180 was selected in relation to other genetic lineages associated with serotype 3 in this period. 6) We found that within the most important serotypes contributing to antimicrobial resistance in adult IPD, which were serotypes 14 and 19A, there were decreases in the proportion of resistant isolates in the post-PCV13 period (Horácio et al., 2016b). Since antimicrobial resistance was highly associated with genotype (Horácio et al., 2016a), these changes in antimicrobial susceptibility might have been associated with changes in the genetic lineages of these two serotypes. We only determined the MLST-defined clonal composition of the isolates causing adult IPD from 2008 to 2011 (Horácio et al., 2016a). It is therefore important to evaluate the clonal composition of serotypes 14 and 19A from 2012 onwards to better understand these changes. 7) We found that half of the isolates expressing serotype 24F in the post-PCV7 period represented CC230 (Horácio et al., 2016a), which was the genetic lineage associated with the expansion of serotype 19A in the post-PCV7 period in Portugal (Aguiar et al., 2010b). Hence, there is a concern that serotype 24F may increase in the post-PCV13 period. This possibility could be evaluated by additional MLST characterization of serotype 24F from 2012

onwards. 8) Almost all isolates expressing serotype 1 in 2008-2011 were from CC306, but two isolates represented CC217 instead of CC306. Representatives of CC217 were associated with high mortality in Africa (Harvey et al., 2011) and therefore the clonal composition of serotype 1 isolates in Portugal should be monitored. 9) In spite of the great diversity of serotypes causing disease in adults, few serotypes were significantly more detected than others (Figures V.S2 and V.S5). It would be important to understand what characteristics promoted the higher frequency of these serotypes and respective genetic lineages when comparing with the other less frequent serotypes. It would also be important to understand why particular serotypes were either associated with adult IPD or adult NIPP (Horácio et al., 2014; Horácio et al., 2018). MLST could be used to evaluate the clonal composition of adult NIPP isolates, as was done with adult IPD (Horácio et al., 2016a). A comparison of adult NIPP and adult IPD clonal compositions may give rise to new questions, which perhaps can be answered by WGS.

Even though co-morbidities increase with increasing age, old age itself was found to be an independent predictor of mortality among patients with IPD (Regev-Yochay et al., 2017). Contradicting the todays accelerated increase in the World population, in Portugal the population is estimated to decrease from 10,3 million in 2015 to 7,5 million in 2080 (INE, 2017). However, the fraction of the population represented by adults ≥ 65 years is estimated to continue to increase until 2040, the moment it will start to decrease due to the inclusion in this age group of people born in the context of low natality rates. If the predictions are correct, the 2,1 million adults ≥ 65 years in 2015 will change to 2,8 million in 2080 (INE, 2017). Therefore, the number of people at high risk for pneumococcal disease is expected to increase in our country during this century, highlighting the importance of continuing epidemiological surveillance of adult pneumococcal disease in Portugal. Since some of the risk factors associated with the development of pneumococcal disease are preventable (e.g. alcohol abuse and smoking), another way to prevent pneumococcal disease, besides vaccination, would be through the adoption of a heathier life style by each individual. Perhaps more campaigns could be done in this regard in the targeted populations.

Supporting Information

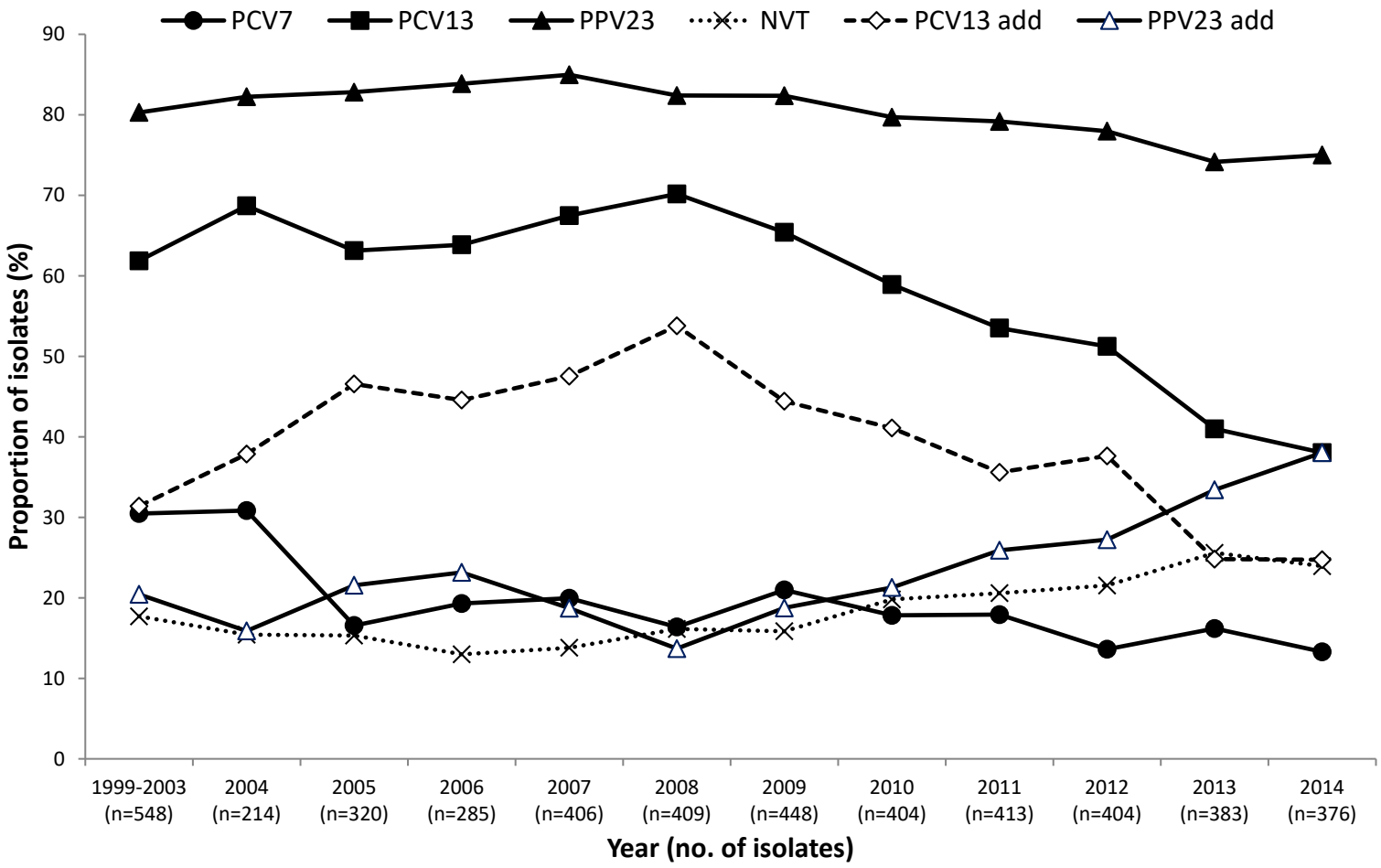


Figure V.S1 Proportion of isolates expressing serotypes included in pneumococcal vaccines causing invasive pneumococcal disease in adult patients (≥ 18 years) in Portugal, 1999–2014. The data up to 2008 were presented previously (Serrano et al., 2004; Aguiar et al., 2008a; Horácio et al., 2012), while the data from 2009 to 2014 were presented in the two studies composing chapter II of this thesis (Horácio et al., 2013; Horácio et al., 2016b).

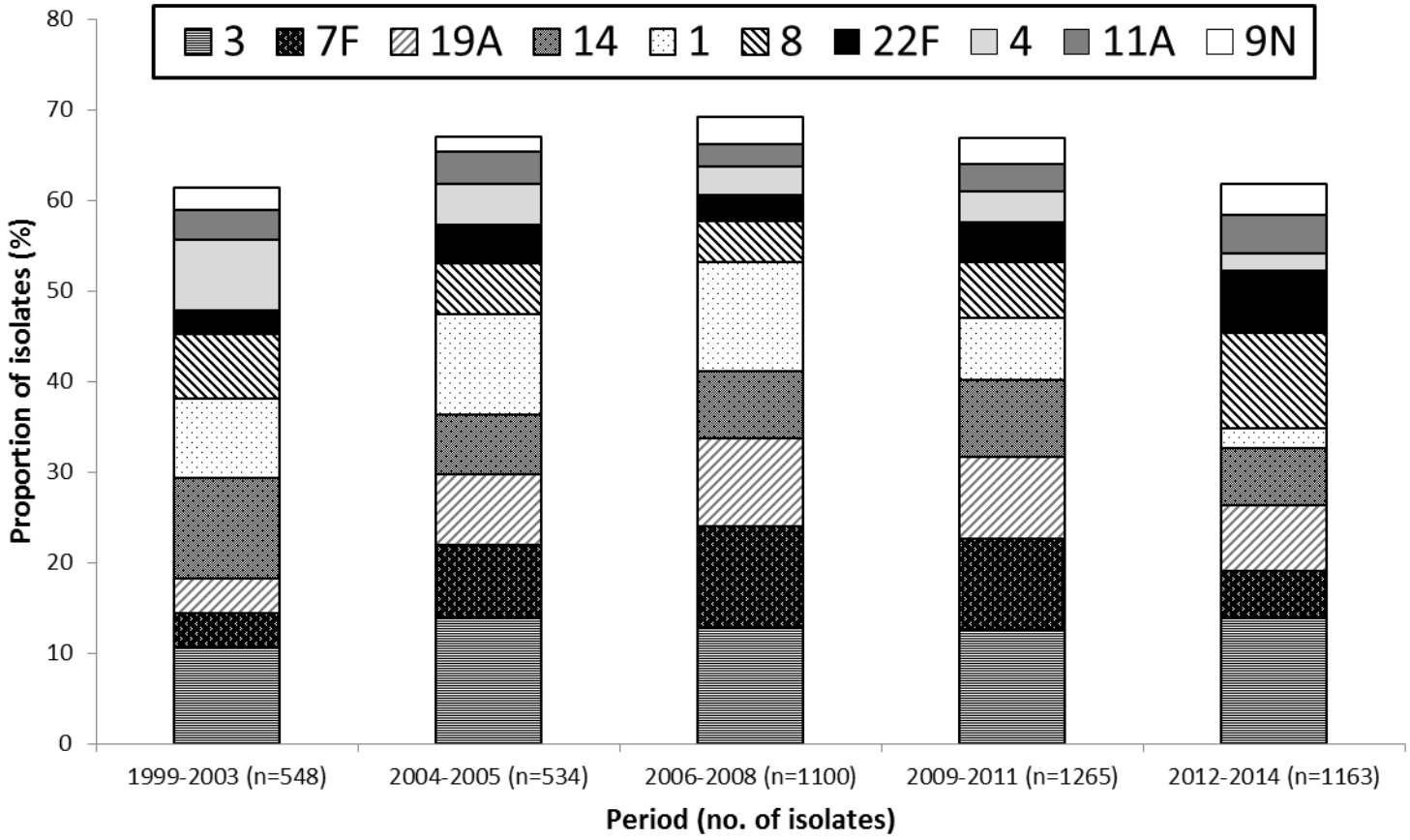


Figure V.S2 - Distribution of the 10 most frequent serotypes causing invasive pneumococcal disease in adult patients (≥ 18 years) in Portugal, 1999–2014. The data up to 2008 were presented previously (Serrano et al., 2004; Aguiar et al., 2008a; Horácio et al., 2012), while the data from 2009 to 2014 were presented in the two studies composing chapter II of this thesis (Horácio et al., 2013; Horácio et al., 2016b).

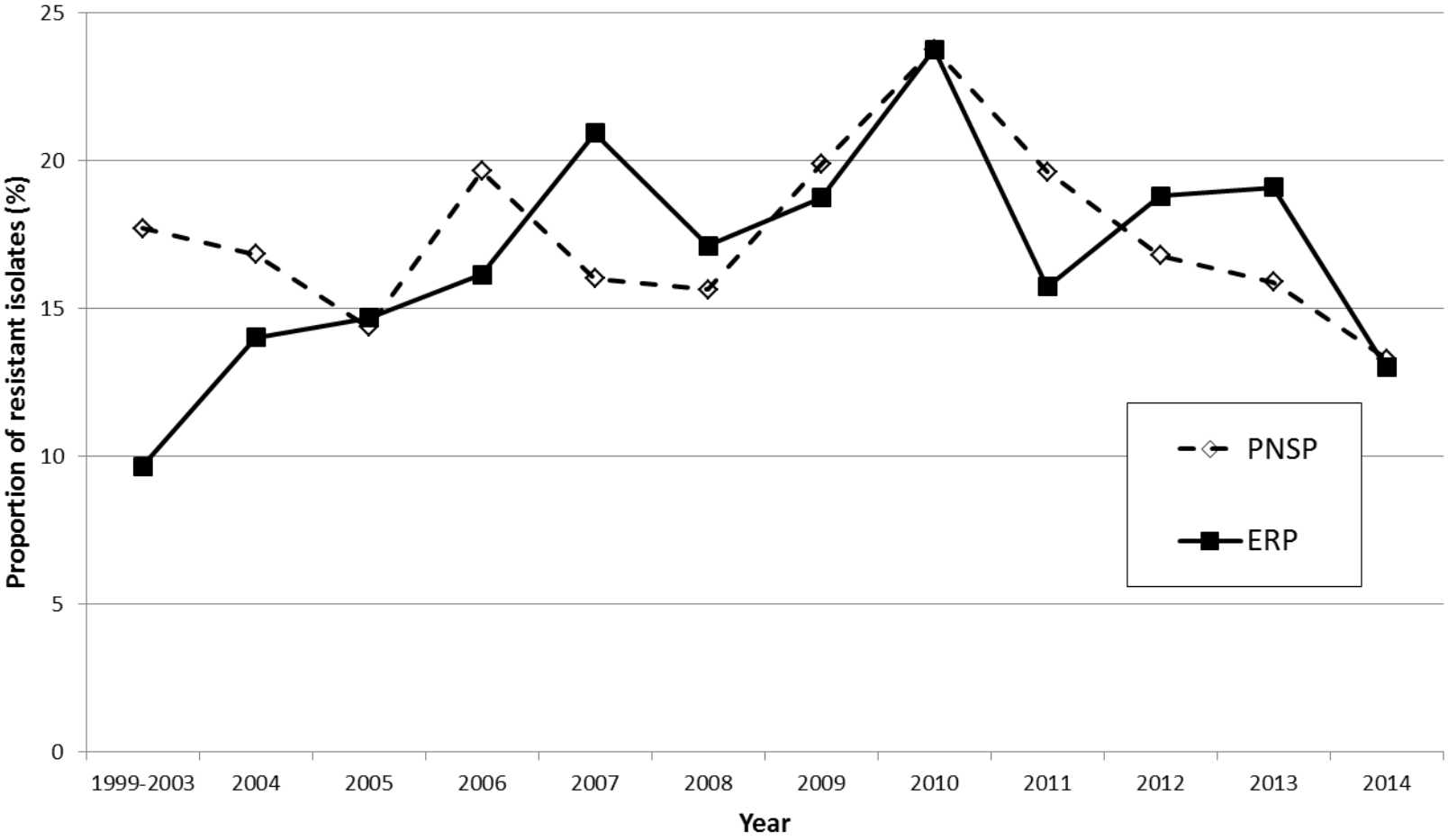


Figure V.S3 – Proportion of penicillin non-susceptible pneumococci (PNSP) and erythromycin resistant pneumococci (ERP) among the isolates causing invasive pneumococcal disease in adult patients (≥ 18 years) in Portugal, 1999–2014. The data up to 2008 were presented previously (Serrano et al., 2004; Aguiar et al., 2008a; Horácio et al., 2012), while the data from 2009 to 2014 were presented in the two studies composing chapter II of this thesis (Horácio et al., 2013; Horácio et al., 2016b).

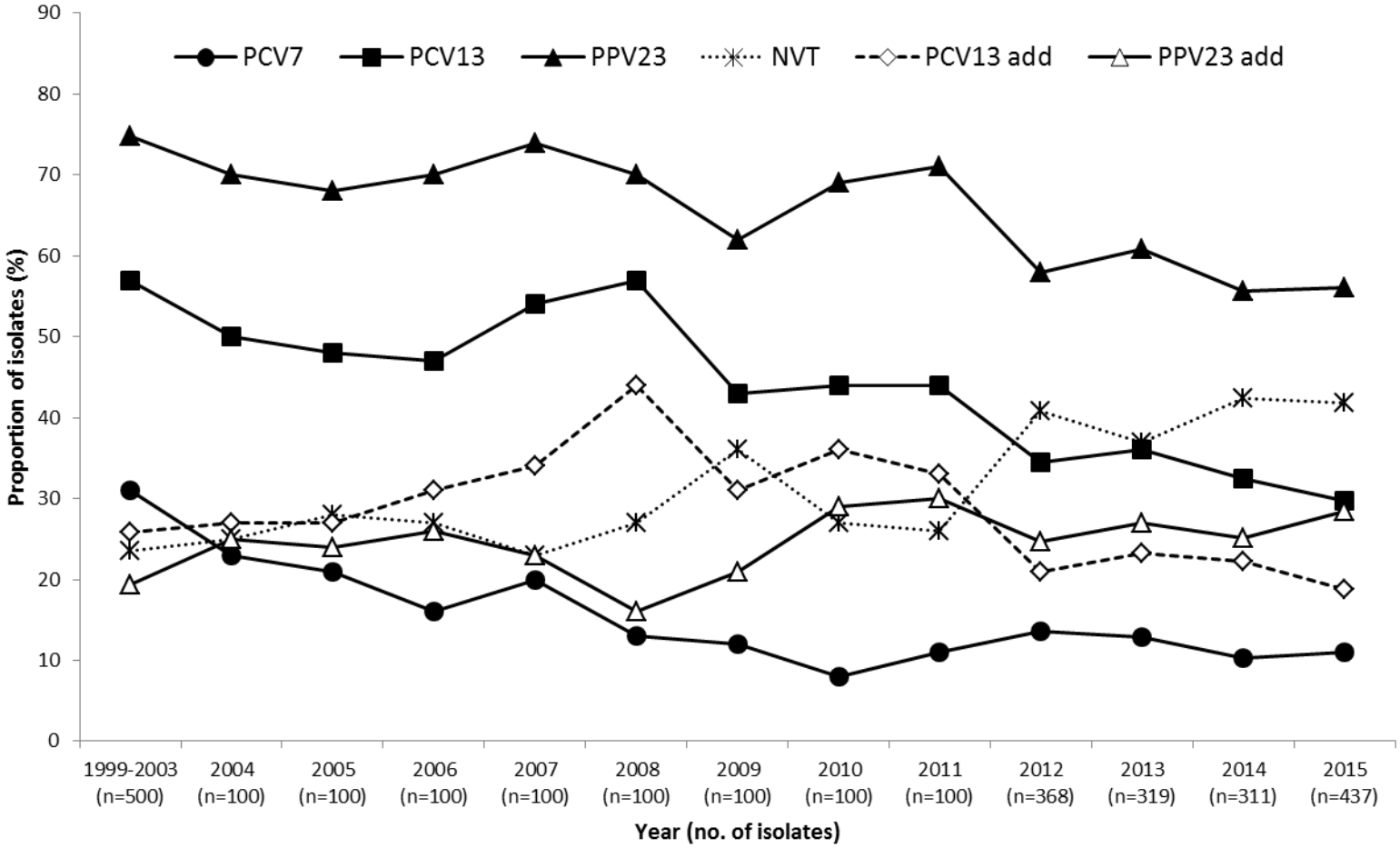


Figure V.S4 - Proportion of isolates expressing serotypes included in pneumococcal vaccines causing non-invasive pneumococcal pneumonia in adult patients (≥ 18 years) in Portugal, 1999–2015. Data from 1999 to 2011 were presented in the first study of chapter IV of this thesis (Horácio et al., 2014), while data from 2012 to 2015 were presented in the second study of chapter IV (Horácio et al., 2018).

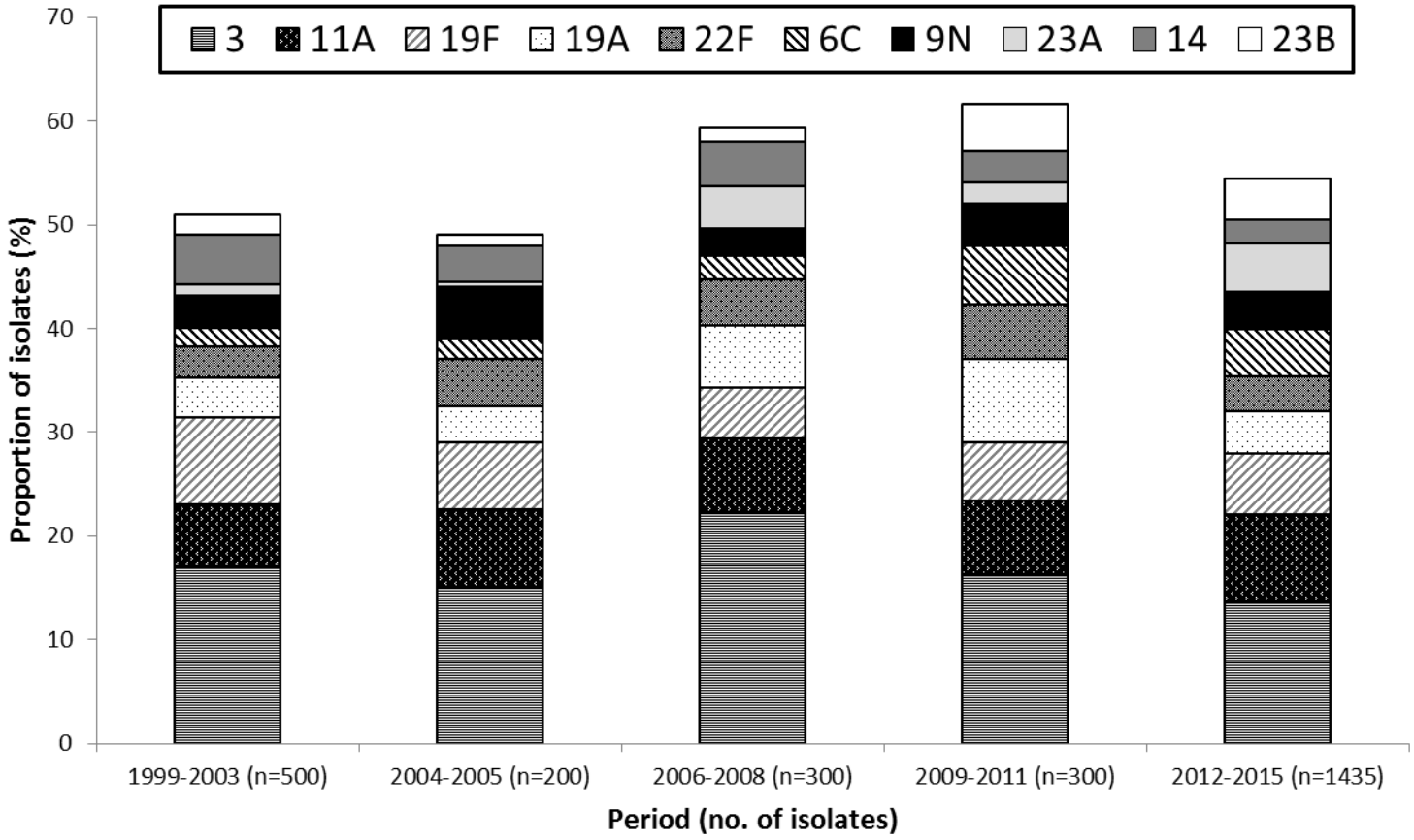


Figure V.S5 - Distribution of the 10 most frequent serotypes causing non-invasive pneumococcal pneumonia in adult patients (≥ 18 years) in Portugal, 1999–2015. Data from 1999 to 2011 were presented in the first study of chapter IV of this thesis (Horácio et al., 2014), while data from 2012 to 2015 were presented in the second study of chapter IV (Horácio et al., 2018).

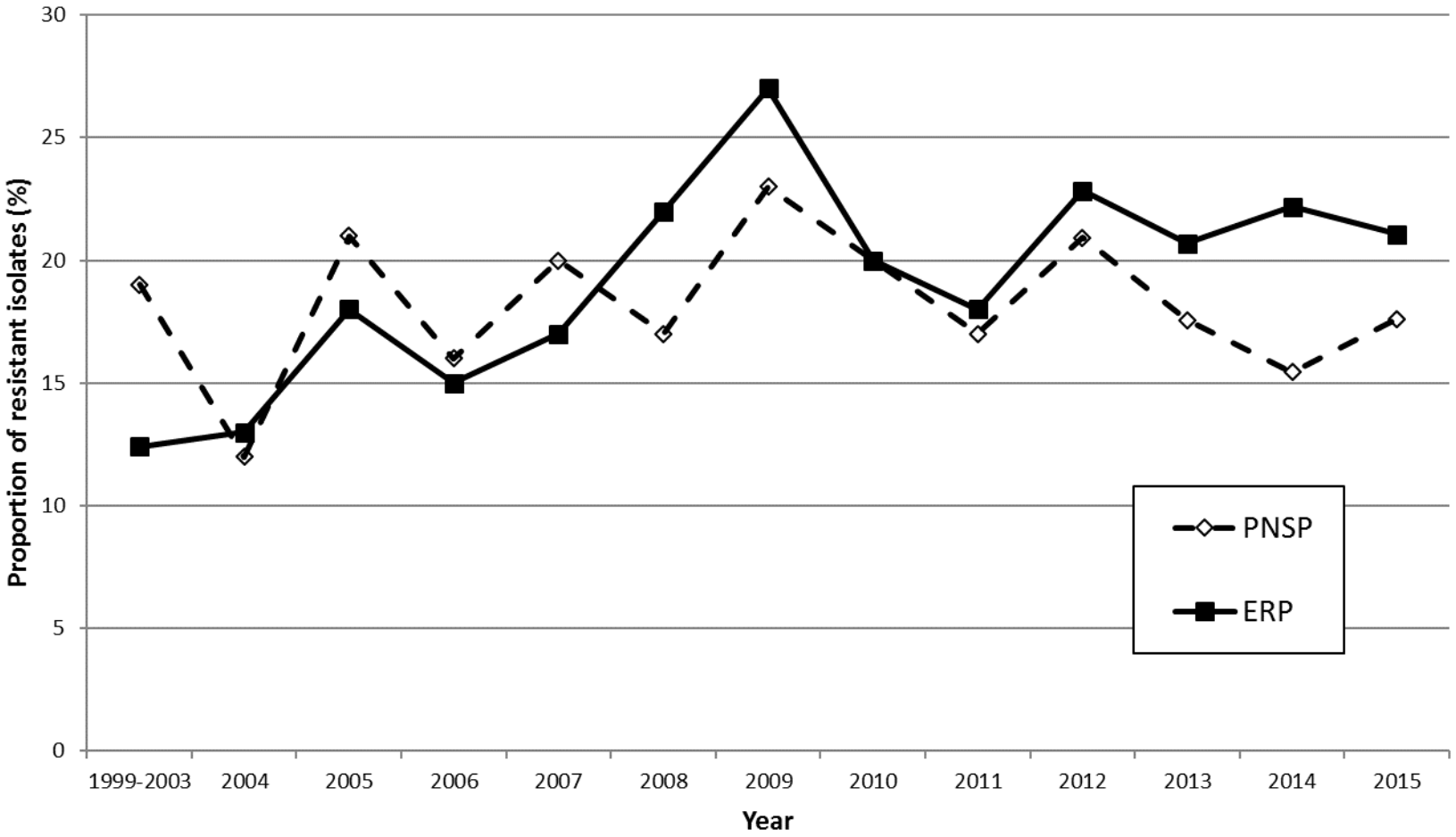


Figure V.S6 – Proportion of penicillin non-susceptible pneumococci (PNSP) and erythromycin resistant pneumococci (ERP) among the isolates causing non-invasive pneumococcal pneumonia in adult patients (≥ 18 years) in Portugal, 1999–2015. Data from 1999 to 2011 were presented in the first study of chapter IV of this thesis (Horácio et al., 2014), while data from 2012 to 2015 were presented in the second study of chapter IV (Horácio et al., 2018).

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The Majority of Adult Pneumococcal Invasive Infections in Portugal Are Still Potentially Vaccine Preventable in Spite of Significant Declines of Serotypes 1 and 5

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Abstract

In Portugal, pneumococcal conjugate vaccines have been administered to children outside of the national immunization plan since 2001. We determined the serotype and antimicrobial susceptibility of 1265 isolates responsible for adult invasive pneumococcal infections (IPD) between 2009 and 2011 and compared the results with previously published data from 1999 to 2008. Serotypes 3 (12.6%), 7F (10.0%), 19A (9.1%), 14 (8.4%), 1 (6.9%) and 8 (6.2%) were the most frequent and together accounted for 53.2% of adult IPD. Serotypes 1 and 5 declined significantly while serotype 34, not included in any vaccine, increased. Taken together, the serotypes included in the 13-valent conjugate vaccine (PCV13) peaked among adult IPD isolates in 2008 (70.2%) and declined since then reaching 53.5% in 2011. The decline in the serotypes included in the 23-valent polysaccharide vaccine since 2007 was also significant but much more modest with 79.2% of the isolates causing IPD in 2011 expressing these serotypes. Since the changes in serotypes causing IPD in adults coincided with the 10-valent and PCV13 introduction in children, it is unlikely that vaccination triggered these changes although it may have accelerated them. The proportion of IPD caused by serotypes included in the 7-valent conjugate vaccine remained stable (19.0%). Both penicillin non-susceptibility and erythromycin resistance increased in the study period, with serotypes 14 and 19A accounting for the majority of resistant isolates.

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Introduction

Streptococcus pneumoniae (pneumococcus) remains a significant cause of morbidity and mortality throughout the world affecting disproportionally the extremes of life. Prevention of these infections in persons ≥2 years belonging to risk groups and particularly among adults ≥65 years has relied on a vaccine including 23 of the 94 capsular polysaccharides known in pneumococci. Although older age is a recognized risk factor for pneumococcal disease, in Europe different countries have distinct recommendations regarding the use of the 23-valent polysaccharide vaccine (PPV23), ranging from the absence of national guidelines, to recommendations of universal or risk group vaccination starting at 60 or 65 years [1]. Perhaps because of an ongoing debate on PPV23 efficacy [2,3,4], in most European countries there is a low overall uptake of PPV23 [5]. In Portugal PPV23 uptake is at the lower end of the spectrum with estimates that approximately 10% of adults ≥65 years are vaccinated [6].

The remarkable efficacy of the seven-valent conjugate vaccine (PCV7) against the serotypes included in its formulation resulted in a sharp decline in the proportion of invasive pneumococcal infections (IPD) caused by these serotypes not only in vaccinated children [7,8,9,10,11,12] but also across the entire population [6]. This "herd effect" is attributed to the reduced transmission of these serotypes from children to adults. In Europe, although there were epidemiological changes in the serotypes causing IPD in the non-vaccinated population in all countries where the vaccine was administered, the large reduction in the overall number of invasive infections in adults observed in the USA was not replicated in countries such as Spain, England and Wales and the Netherlands [10,12,13] where significant increases in non-vaccine serotypes (NVT) occurred.

Since the PCV7 serotypes represented a significant fraction of resistant isolates, vaccination was also anticipated to affect resistance. However, the effect of PCV7 on antibiotic resistance in Europe was variable. While a decrease in penicillin non-susceptibility was noted in all countries among isolates responsible

for pediatric IPD in the post-PCV7 period [7,13,14], such decline was not apparent in adults in Portugal and Spain [6,13,14].

Serotype 19A has consistently been identified as a dominant non-vaccine serotype but other emerging non-vaccine serotypes differ between geographic locations and also between age groups [6,7,8,9,14]. Even within serotype 19A, different genetic lineages emerge in different geographic locations [15]. These data highlight the importance of the characteristics of the local pneumococcal population and of local selective forces in conditioning the outcomes of vaccination [16].

On the other hand, it is known that serotypes responsible for IPD may have significant temporal variations in the same geographic region as documented in Spain and Denmark [17,18], even with limited antibiotic selective pressure and in the absence of PCV use. In addition, the divergent prevalence of the various serotypes in different geographic regions also conditions the potential benefits of vaccination. A much lower prevalence of serotype 1 IPD is documented in the USA than elsewhere [6,9,14,18,19,20]. Although serotype 1 is frequently associated with outbreaks and significant yearly variations of the proportion of IPD caused by this serotype are documented, a considerable fraction of IPD was consistently caused by this serotype in the last decades in Europe [17,18].

Two new pneumococcal conjugate vaccine (PCV) formulations are now commercially available and used in children. A 10-valent formulation (PCV10) including, in addition to the PCV7 serotypes, serotypes 1, 5 and 7F and a 13-valent conjugate vaccine (PCV13), including all PCV10 serotypes plus serotypes 3, 6A and 19A. The introduction of these vaccines into clinical practice has the potential to once again change the characteristics of pediatric IPD, with initial data showing the capacity of PCV13 to blunt or even reverse the rise of some of the most successful serotypes that have emerged as causes of pediatric IPD since the introduction of PCV7 [21,22,23].

PCV13 was recently licensed for use in adults ≥50 years and this was soon followed by a recommendation in the USA for its use in adults with immunocompromising conditions [24]. Approval of PCV13 for adults was based on immunogenicity studies and the results of a large study that is currently underway in the Netherlands [25] comparing it to placebo for the prevention of vaccine-serotype community acquired pneumonia in adults are expected to become available in late 2013. The observed benefits of conjugate vaccines in children launched a discussion about the potential benefits of vaccinating the adult population with these vaccines instead of PPV23 [2,3,4,26]. Independently of the immunological arguments, the potential benefits of adult vaccination with either PCV13 or PPV23 are a moving target since secular trends in pneumococcal serotypes and the herd effect provided by PCV7, and now also hoped for the use of PCV13 in children, would be expected to reduce the importance of the serotypes included in conjugate vaccines in adult IPD.

In Portugal PCVs were not included in the National Immunization Plan but there has been a steady increase in PCV7 uptake since 2001, reaching 75% of children ≤2 yrs in 2008 [14]. The expanded valency PCVs for childhood vaccination – PCV10 and PCV13– became available in mid-2009 and early-2010, respectively. In previous studies, we showed that significant changes in the serotypes causing IPD in children followed PCV7 availability [7,14] and that there was evidence for a herd effect in the adult population [6,14]. This study aimed at documenting the continued changes on serotype distribution and antimicrobial resistance in different adult groups and evaluating the proportion of potentially vaccine preventable adult IPD in Portugal immediately prior to the approval in January 2012 of PCV13 use in adults >50 yrs.

Materials and Methods

Ethics Statement

Case reporting and isolate collection were considered to be surveillance activities and were exempt from evaluation by the Review Board of the Faculdade de Medicina da Universidade de Lisboa.

Bacterial Isolates

Since 1999, the Portuguese Group for the Study of Streptococcal Infections has monitored pneumococci causing invasive infections in Portugal. This is a laboratory-based surveillance system, in which 30 microbiology laboratories throughout Portugal are asked to identify all isolates responsible for IPD and to send them to a central laboratory for characterization. A case of invasive disease is defined by an isolate of *S. pneumoniae* recovered from a normally sterile body site such as blood or CSF. Although the laboratories were contacted periodically to submit the isolates to the central laboratory, no audit was performed to ensure compliance, which may be variable in this type of study. Isolates recovered up to 2008 were previously characterized [6,14,27]. Only isolates recovered from adult invasive infections, i.e. recovered from patients ≥18 yrs, between 2009 and 2011 were included in the present study. One isolate from each patient in each year was considered. All strains were identified as *S. pneumoniae* by colony morphology and hemolysis on blood agar plates, optochin susceptibility and bile solubility.

Serotyping and Antimicrobial Susceptibility Testing

Serotyping was performed by the standard capsular reaction test using the chessboard system and specific sera (Statens Serum Institut, Copenhagen, Denmark). Serotypes were grouped into conjugate vaccine serotypes, i.e., those included in PCV13 (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 19A, 23F) and that comprise all the serotypes found in the lower valency vaccines, those included in PPV23 (all serotypes included in PCV13 except 6A and serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F), and non-vaccine serotypes (NVT). Etest strips (AB Biodisk, Solna, Sweden) were used to determine the MICs for penicillin and cefotaxime. In 2008, the CLSI changed the recommended breakpoints used to interpret MIC values. Unless otherwise stated we have used the CLSI-recommended breakpoints prior to 2008 [28] as epidemiological breakpoints that allow the comparison with previous studies. According to these recommendations, intermediate level penicillin resistance was defined as MIC 0.12–1.0 μg/ml and high level resistance as MIC ≥2.0 μg/ml. Isolates that fell into either of these classes were designated penicillin non-susceptible. Susceptibility to cefotaxime was defined as MIC ≤1.0 μg/ml for non-meningitis cases and an MIC ≤0.5 μg/ml for meningitis cases.

Isolates were further characterized by determining their susceptibility to erythromycin, clindamycin, vancomycin, linezolid, tetracycline, levofloxacin, trimethroprim-sulfamethoxazole and chloramphenicol by the Kirby-Bauer disk diffusion technique, according to the CLSI recommendations and interpretative criteria [29].

Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin according to a previously published procedure [30]. Simultaneous resistance to erythromycin and clindamycin defines the MLS_B phenotype (resistance to macrolides, lincosamides and streptogramin B) while non-susceptibility only to erythromycin indicates the M phenotype.

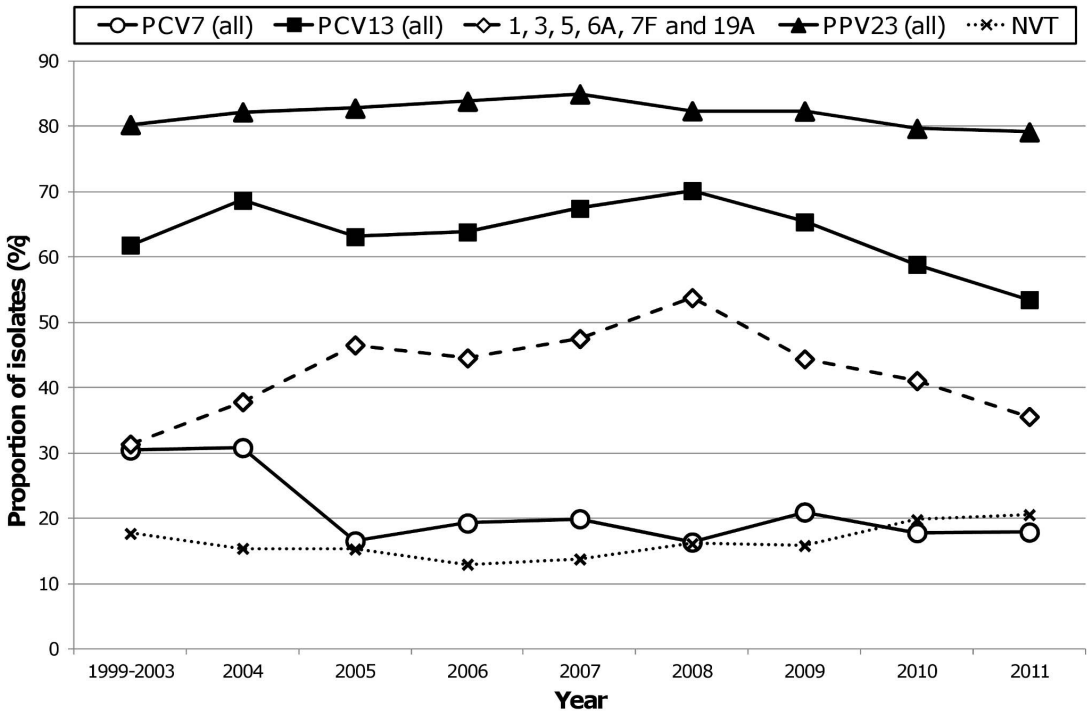

Figure 1. Proportion of isolates expressing serotypes included in pneumococcal vaccines causing invasive infections in adults in Portugal (1999–2011). The data up to 2008 were presented previously [6,14,27]. The period of 1999–2003, previously identified as the pre-PCV7 period [14], was analyzed together.
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The prevalence of the various serotypes was compared with already published data from 1999–2008 [6,14,27]. We established previously that no significant changes in serotype distribution occurred until 2003 in adult IPD and have therefore considered 1999–2003 as the pre-vaccine period [14].

Statistical Analysis

Simpson's index of diversity (SID) and respective 95% confidence intervals ($CI_{95\%}$) was used to measure the population diversity [31]. Adjusted Wallace (AW) coefficients were used to compare two sets of partitions [32]. The calculation of these indices was done using the online tool www.comparingpartitions.info. Differences were evaluated by the Fisher exact test and the Cochran-Armitage test was used for trends with the false discovery rate (FDR) correction for multiple testing [33]. A $p<0.05$ was considered significant for all tests.

Results

Isolate Collection

Between 2009 and 2011 a total of 1265 isolates were recovered from normally sterile sites: 448 in 2009, 404 in 2010 and 413 in 2011. Isolates were recovered from blood (n = 1121, 88.6%), CSF (n = 97, 7.7%), pleural fluid (n = 30, 2.4%), peritoneal fluid (n = 10, 0.8%) and other normally sterile sites (n = 7, 0.5%). Regarding age distribution, 353 isolates (27.9%) were recovered from patients 18–49 yrs, 272 (21.5%) from patients 50–64 yrs and 640 (50.6%) from patients ≥65 yrs.

The 1265 isolates recovered in 2009–2011 are in line with the 1100 isolates recovered in 2006–2008 and reported previously [6]. This suggests that the surveillance network is stable and that no major changes are affecting IPD reporting in the two periods. However, although unlikely, we cannot completely exclude the possibility that there was an increase in reporting that may have compensated for a potential decrease in IPD incidence.

Serotype Distribution

We detected 50 different capsular types among the 1265 isolates. The most frequent, that accounted for 53.2% of all adult IPD, were serotypes 3 (n = 160, 12.6%), 7F (n = 126, 10.0%), 19A (n = 115, 9.1%), 14 (n = 106, 8.4%), 1 (n = 87, 6.9%) and 8 (n = 79, 6.2%). During the study period (2009–2011), the only significant changes found in individual serotype prevalence after FDR correction were of serotype 1, that decreased from 10.7% to 4.1% (Cochran-Armitage test of trend $p<0.001$), serotype 5, that decreased from 2.0% to 0% (Cochran-Armitage test of trend $p = 0.003$) and serotype 34, that did not cause any invasive infections in 2009 and 2010 but was detected in 1.9% of the isolates causing IPD in 2011 (Cochran-Armitage test of trend $p<0.001$).

Fig. 1 shows the evolution of vaccine preventable IPD between 1999 and 2011. For the period 1999–2003, defined previously as the pre-PCV7 period [14], the results were averaged over the entire period. After the significant decline of IPD caused by PCV7 serotypes between 2004 and 2005, from 30.8% to 16.5% ($p<0.001$), a steady and low prevalence was seen until 2011. As previously documented [6], in spite of the decrease of PCV7 serotypes, the increase in serotypes 1, 19A and 7F resulted in an overall increase in PCV13 serotypes in the post-PCV7 period, from 61.9% in 1999–2003 to 70.2% in 2008 (Cochran-Armitage test of trend $p = 0.014$). However, 2008 was an inflection point (Fig. 1) and the proportion of isolates presenting PCV13 serotypes started to decline from then onwards such that in 2011 only 53.5% of the isolates presented PCV13 serotypes (from 2008 to 2011, Cochran-Armitage test of trend $p<0.001$). This change was mainly

driven by a decrease in prevalence of serotypes 1 and 5 from 13.5% and 2.9% in 2008 to 4.1% and 0% in 2011, respectively (Cochran-Armitage test of trend $p<0.001$ for both, significant after FDR) (Table S1).

The proportion of PPV23 serotypes also increased slightly but non-significantly up to 2007. From 2007 onwards there was a slight but significant decrease in the proportion of IPD caused by PPV23 serotypes, from 85.0% in 2007 to 79.2% in 2011 (Cochran-Armitage test of trend $p = 0.018$). Initially this decline occurred in spite of the increase in PCV13 serotypes that peaked in 2008. Later, the decline of PCV13 serotypes was opposed by an important increase in the proportion of IPD caused by the additional serotypes found in PPV23 but absent from PCV13, from 13.7% in 2008 to 25.9% in 2011 (Cochran-Armitage test of trend $p<0.001$). When looking individually at these serotypes, although several increased in frequency, only serotype 8 increased significantly from 3.7% in 2008 to 8.0% in 2011 (Cochran-Armitage test of trend $p<0.002$, significant after FDR).

Fig. 2 shows the distribution of the individual serotypes included in the conjugate vaccines, stratified by the age group of the patients. Fig. 3 shows the distribution of the additional serotypes found in PPV23 that are not included in the conjugate vaccines. To analyze the serotype diversity within each age group, SIDs were calculated. The serotypes of the isolates causing invasive infections in any of the age groups considered were highly diverse (18–49 yrs [SID: 0.939, $CI_{95\%}$: 0.930–0.947]; 50–64 yrs [SID: 0.949, $CI_{95\%}$: 0.941–0.958]; ≥65 yrs [SID: 0.934, $CI_{95\%}$: 0.926–0.943]). The only significant difference was a higher diversity of serotypes in the 50–64 yrs age group relative to ≥65 yrs age group ($p = 0.013$). A similar analysis was performed for determining the serotype diversity in each study year but no significant changes occurred between 2009 and 2011 nor were changes in diversity noted between the 2004–2008 period and 2009–2011 (data not shown).

Although the frequency of each serotype varies according to the age groups considered, only for serotypes 1, 3, 8 and 19A were these differences significant. While the frequency of serotype 1 decreases with age (18–49 yrs –11.0%, 50–64 yrs –7.7%, ≥65 yrs –4.2%; Cochran-Armitage test of trend $p<0.001$), the frequency of serotype 3 increases with age (18–49 yrs –7.6%, 50–64 yrs –10.7%, ≥65 yrs –16.3%; Cochran-Armitage test of trend $p<0.001$). For serotypes 8 and 19A a trend with age was not evident. However, serotype 8 was more frequent in the youngest group (18–49 yrs –10.5%) than in either of the oldest age groups (50–64 yrs –4.8%, $p = 0.011$ and ≥65–4.5%, $p<0.001$), and serotype 19A showed a higher prevalence in older adults (≥65 yrs; 10.5%) than in younger adults (18–49 yrs; 5.9%, $p = 0.019$). Taking together the three years of the study (2009–2011), the proportion of IPD caused by the serotypes included in both conjugate vaccines and in PPV23 was roughly the same in all age groups considered (Table 1).

When analyzing individual serotypes with three or more CSF isolates, a positive association with CSF was found for serotypes 6B ($p = 0.002$), 16F ($p = 0.009$) and 19F ($p = 0.002$), all significant after FDR (Table S2).

Antimicrobial Susceptibility

Resistance to the tested antimicrobials is summarized in table 2 and figures 2 and 3. Overall 266 isolates (21.0%) were penicillin non-susceptible pneumococci (PNSP) –205 (16.2%) expressed low level resistance (MIC = 0.12–1.0) and 61 (4.8%) high level resistance (MIC ≥2 μg/ml). Considering current CLSI breakpoints for parenteral penicillin where susceptibility is defined as MIC <0.06 μg/ml for meningitis cases [29], 17 isolates (17.5%) from CSF would have been considered resistant and 27 isolates (2.3%) from non-meningitis cases would have been considered non-susceptible –24 (2.1%) intermediately resistant and 3 (0.3%) fully resistant. Erythromycin resistant pneumococci (ERP) accounted for19.4% of the isolates (n = 245), of which 194 isolates (79.2%) expressed the MLS_B phenotype and 51 (20.8%) the M phenotype. All isolates were susceptible to vancomycin and linezolid. The simultaneous expression of erythromycin resistance and penicillin non-susceptibility (EPNSP) was found in 13.0% of the isolates.

Resistance to penicillin, erythromycin and clindamycin increased with the age group considered (table 2). While the increase in erythromycin and clindamycin were statistically supported (Cochram-Armitage test of trend, $p = 0.035$ and $p = 0.039$, respectively) the increase in penicillin non-susceptibility was not ($p = 0.057$). For the other tested antimicrobials no significant differences were noted. The proportions of PNSP and ERP in 2009–2011 were higher than those previously reported (Figure S1). The proportion of PNSP increased from being previously stable (from an average value of 16.7% in 1999–2008 to 21.0% in 2009–2011, $p = 0.002$). On the other hand, there was a consistent increase in the proportion of ERP since PCV7 introduction (from 1999–2003 to 2011, Cochran-Armitage test of trend $p<0.001$). Although the overall proportion of ERP has been increasing, when analyzing changes within the study period a significant decrease was noted from 2010 to 2011, from 23.8% to 15.7% ($p = 0.005$). Similarly, the proportion of PNSP also decreased from 23.8% in 2010 to 19.6% in 2011, but this change was not statistically significant ($p = 0.174$).

The correlation between serotype and antimicrobial resistance was high. The AW for serotype and penicillin non-susceptibility was 0.539 ($CI_{95\%}$ 0.476–0.601), higher than the AW for serotype and erythromycin resistance (0.403, $CI_{95\%}$ 0.336–0.470). Together, serotypes 19A and 14 contributed greatly to penicillin non-susceptibility (59.0%) and to erythromycin resistance (53.1%) (Fig. 2). Serotypes included in PCV7 represented 47.4%, 36.7% and 35.8% of PNSP, ERP and EPNSP, respectively, while serotypes included in PCV13 constituted 76.7%, 71.4% and 72.1%, respectively. Considering the serotypes included in PPV23, only a modest increase relative to the PCV13 serotypes is noted (80.2%, 75.1% and 74.5%, of PNSP, ERP and EPNSP, respectively) since most of the remaining resistant isolates express NVTs (Fig. 3). Among the EPNSP expressing NVTs, serotypes 6C (45.2%) and 15A (28.6%) were dominant.

Discussion

The effect of childhood vaccination in the distribution of IPD serotypes in adults was always found to be delayed in relation to the effects seen in children in the countries where the vaccine is available [12,14,34]. Given this prior experience with PCV7, the introduction of PCV13 in childhood vaccination in early 2010 in Portugal would only be expected to have an effect in the distribution of serotypes causing IPD in adults around 2011. If one considers the serotypes common to PCV10, then the introduction of PCV10 in childhood vaccination in mid-2009 would raise the possibility that an effect on these serotypes could occur earlier. However, the significant increase in adult IPD in the post-PCV7 period of serotypes 1, 7F (common to both PCVs) and 19A (exclusively found in PCV13) peaked in 2008 [6]. While serotypes 7F and 19A remained stable from then onwards, the number of serotype 1 isolates started to decline in 2009 (Table S1). This was accompanied by a significant reduction of serotype 5 and a strong reduction of serotype 6A (not included in PCV10) in the

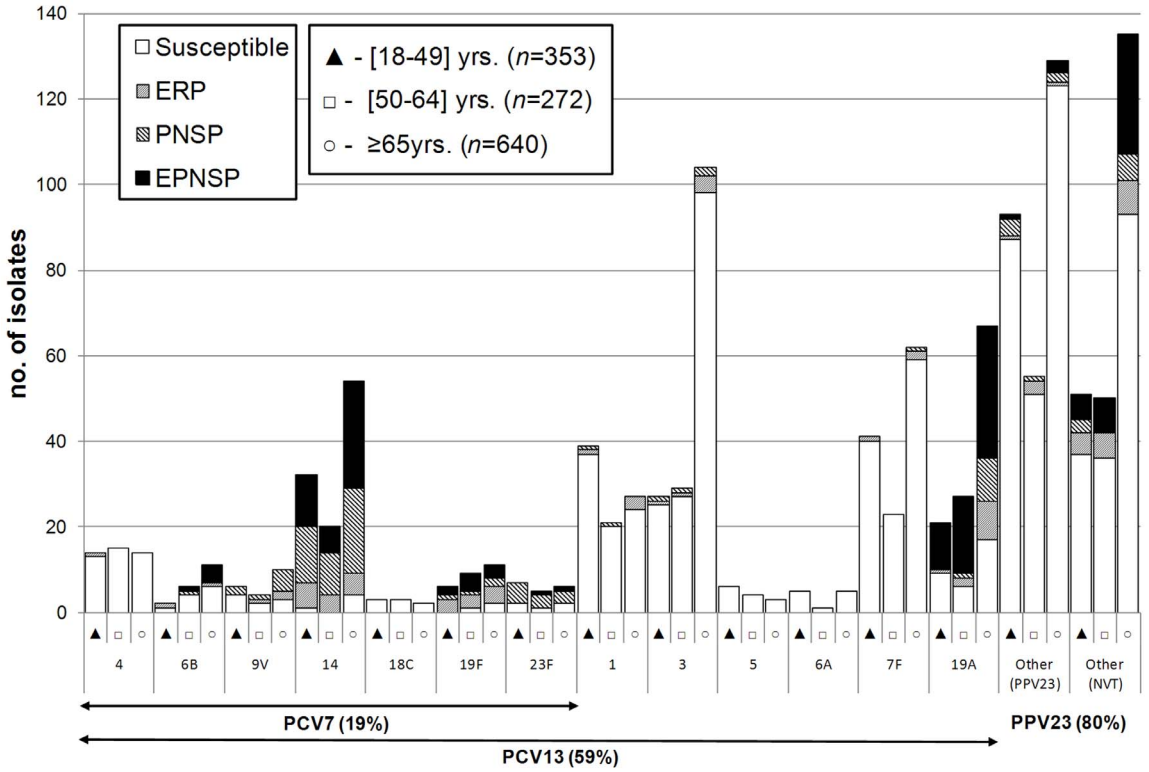

Figure 2. Number of isolates expressing serotypes included in conjugate vaccines causing invasive infections in Portugal (2009–2011). The number of isolates expressing each serotype in each of the age groups considered is indicated. Isolates recovered from patients 18 to 49 yrs are indicated by black triangles. Isolates recovered from patients 50 to 64 yrs are indicated by open squares. Isolates recovered from patients ≥65 yrs are indicated by open circles. Isolates presenting both erythromycin resistance and penicillin non-susceptibility (EPNSP) are represented by closed black bars. Penicillin non-susceptible isolates (PNSP) are indicated by dark hatched bars. Erythromycin resistant pneumococci(ERP) are indicated by light hatched bars. Isolates susceptible to both penicillin and erythromycin are represented by white open bars. The serotypes included in each of the conjugate vaccines are indicated by the arrows. NVT – non-vaccine serotypes, i.e., serotypes not included in any of the currently available vaccines (PCV13 and PPV23). Twenty-eight NVT were detected representing 236 isolates as follows: 6C (n = 36); 23A (n = 20); 12B (n = 19); 16F (n = 18); 23B and 33A (n = 16 each); 15A and 29 (n = 15 each); 24F (n = 13); non-typable (n = 10); 31, 34 and 35F (n = 8 each); 7C, 25A and 35B (n = 5 each); 21 (n = 4); 18A (n = 3); 13 and 17A (n = 2 each); 7A, 11C, 15F, 24A, 25F, 28A, 28F and 37 (n = 1 each).
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same period, that were responsible for the overall fall in PCV13 serotypes (Fig. 1). The fact that the decline started in 2009, before PCV13 introduction and shortly after PCV10 became available, strongly argues that the changes seen here were not triggered by the use of PCVs, although they may have been accelerated by PCV use.

Serotype 3 remains the most prevalent serotype in adult IPD after PCV7 introduction and continued to be significantly associated with older adults [6]. Similarly, serotype 7F remains the second most frequently identified serotype. Although serotypes 3 and 7F were not as frequent, these were also important among IPD in children [7] and were commonly found among patients with pleural parapneumonic effusion of all ages in the same period (unpublished data), attesting to their virulence. In spite of their continued dominance, both serotype 3 and 7F isolates remain mostly susceptible to all tested antimicrobials (Fig. 1), as previously described in Portugal and elsewhere [18,35].

The increase in serotype 19A as a cause of IPD in both children [7] and adults [6] plateaued in the study period in adults with the overall proportion of IPD isolates expressing this serotype remaining stable at around 9%. Serotype 19A as a whole, did not have an enhanced propensity to cause invasive infections [36], but particular lineages within this serotype were found to have different preferences in their association with the human host [15]. In Portugal it was shown that the lineage that was expanding was associated with antimicrobial resistance [15], as was also seen here (Fig. 2). While no association with particular adult age groups was seen previously for serotype 19A IPD, this serotype was now significantly associated with older adults (≥65 yrs) rather than younger adults (18–49 yrs). This could be driven in part by a higher antimicrobial consumption in this age group, a trend that may be emerging in developed countries [37]. However, in Norway the post-PCV7 rise of serotype 19A was mostly dominated by a penicillin susceptible clone [38], suggesting that selection for antimicrobial resistance alone cannot explain the post-PCV7 rise of this serotype.

Despite 10 years of PCV7 use in children, serotype 14 was still responsible for a significant fraction of IPD in all adult age groups (Fig. 2) in contrast to what happens elsewhere, where more significant reductions of serotype 14 in adult IPD followed PCV7 use in children [12,34]. Serotype 14 isolates were mostly resistant to either erythromycin or penicillin or both (101/106, 95%) (Fig. 2). A high proportion of penicillin non-susceptibility, erythromycin resistance and erythromycin and penicillin non-susceptibility has been a characteristic of serotype 14 isolates since before PCV7 introduction [14,27,35], a feature that was accentuated in the post-PCV7 period in both pediatric and adult IPD [6,7].

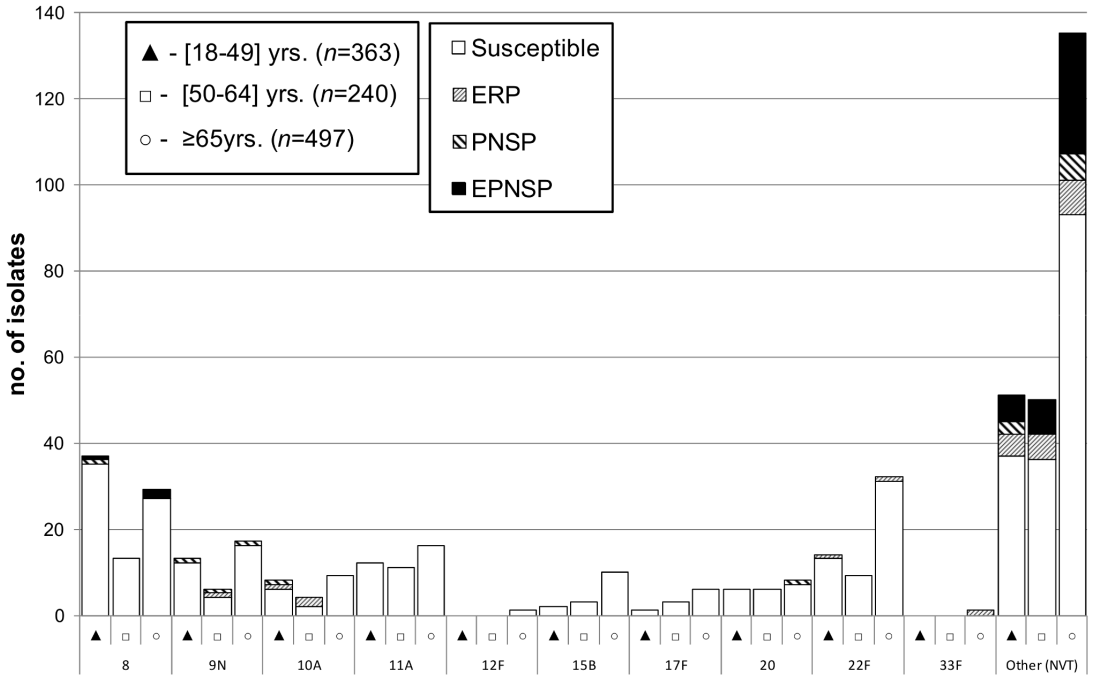

Figure 3. Number of isolates expressing serotypes present in the 23-valent polysaccharide vaccine but not included in conjugate vaccines causing invasive infections in Portugal (2009–2011). See the legend of figure 1. Out of the 11 serotypes present in the 23-valent polysaccharide vaccine PPV23 but absent from the 13-valent conjugate vaccine PCV13, serotype 2 was not found in our collection.
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Serotype 1 traditionally accounts for a higher proportion of pediatric IPD in Europe than in North America. In Portugal serotype 1 was the second most important serotype in adult IPD between 2006–2008 [6]. In neighboring Spain, outside the Madrid area where PCV7 was available but not included in the national immunization plan similar to Portugal, an increased importance of serotype 1 in IPD both in the group targeted for PCV7 vaccination as well as in older children and adults was also documented [39]. The decline in the importance of serotype 1 as a cause of IPD with age in adults reported previously [6] was also seen in this period. However, serotype 1 dropped from the second most frequent overall cause of IPD in adults to fifth. As discussed above, the trigger of this decline cannot be solely attributed to a possible herd effect of PCV use in children since it started before vaccination of children with the expanded valency PCVs that include this serotype. Since serotype 1 remains mostly susceptible to antimicrobials (Fig. 2), the continued pressure of antimicrobial use could be invoked to explain this reduction. However, this does not seem plausible since serotypes 3, 4 and 7F, all also included in PCV13

Table 1. Isolates expressing serotypes included in pneumococcal vaccines (2009–2011).

		No. isolates (%)		
		18–49 yrs	50–64 yrs	≥65 yrs
	2009	24 (18.2)	21 (22.8)	49 (21.9)
PCV7	2010	22 (19.6)	20 (24.7)	30 (14.2)
	2011	24 (22.0)	21 (21.2)	29 (14.1)
	2009	80 (60.6)	63 (68.5)	150 (67.0)
PCV13	2010	65 (58.0)	48 (59.3)	125 (59.2)
	2011	64 (58.7)	56 (56.6)	101 (49.3)
	2009	104 (78.8)	78 (84.8)	187 (83.5)
PPV23	2010	97 (86.6)	66 (81.5)	159 (75.4)
	2011	96 (88.1)	77 (77.8)	154 (75.1)

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Table 2. Antimicrobial resistance of the isolates responsible for invasive infections in adults (2009–2011).

	No. resistant isolates (%)[a]		
	18–49 yrs	50–64 yrs	≥65 yrs
	(n = 353)	(n = 272)	(n = 640)
PEN	62 (17.6)	58 (21.3)	146 (22.8)
MIC_{90}	1	1	1
MIC_{50}	0.023	0.023	0.023
CTX	14 (4.0)	12 (4.4)	24 (3.8)
MIC_{90}	0.75	0.75	0.75
MIC_{50}	0.023	0.023	0.023
LEV	0 (0)	0 (0)	2 (0.3)
ERY	53 (15.0)	58 (21.3)	134 (20.9)
CLI	40 (11.3)	51 (18.8)	108 (16.9)
CHL	7 (2.0)	11 (4.0)	11 (1.7)
SXT	68 (19.3)	62 (22.8)	117 (18.3)
TET	49 (13.9)	51 (18.8)	118 (18.4)

[a]PEN – penicillin; CTX – cefotaxime; LEV – levofloxacin; ERY – erythromycin; CLI – clindamycin; CHL – chloramphenicol; SXT – trimethoprim/sulphamethoxazole; TET – tetracycline. All isolates were susceptible to vancomycin and linezolid.
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and mostly susceptible to antimicrobials similarly to serotype 1 isolates, remain important and stable serotypes in IPD in adults in Portugal in the post-PCV7 period. Serotypes 1 and 7F were found to have an enhanced invasive disease potential [36] and were thus candidates to increase in prevalence in IPD in the post-PCV7 period, as indeed happened [6]. The decline in serotype 1 could be due to unexplained temporal trends that have been known to affect this serotype [17,18]. These temporal trends may have subsequently been accelerated by PCV use.

Two other serotypes with enhanced invasive disease potential – serotypes 5 and 8 [36]– showed opposite trends. Serotype 5 was shown to cause outbreaks in open communities [40] and a significant increase in cases had been noted in 2008, although we have no evidence that these cases correspond to an outbreak. The subsequent decline of serotype 5 isolates could then be the natural dynamics of a putative outbreak. The suggestion that PCV use could have contributed to the decline of serotype 5 is supported by the observation that 2011 is the only year since surveillance started in 1999 when no isolates expressing serotype 5 were detected among IPD cases in adults. Serotype 8 on the other hand is a non-PCV serotype that has been increasing since 2008 and that is now the sixth most frequent serotype in IPD in adults (Fig. 3). Serotype 34 is a NVT that increased in the study period, although it is responsible for a modest number of cases. Although this serotype as a whole was associated with carriage, different lineages expressing this serotype showed distinct capacities to cause invasive disease [36]. It is therefore possible that its increase in IPD documented here is driven by a limited expansion of particularly virulent lineages. The other non-PCV serotypes that are among the ten most frequently found in adult IPD (Fig. 3) have all increased in frequency, although this was not statistically supported, and included the PPV23 serotypes 22F, 11A and 9N and the NVT serotype 6C. The latter was also notable for being frequently resistant to both erythromycin and penicillin. Serotypes 6C and 22F were also found among the most frequent in adult IPD in Canada in 2010 [34]. Since these are not covered by currently available PCVs, these serotypes may emerge as important causes of adult IPD in the post-PCV era.

In spite of the large reduction in the number of PCV7 serotypes, the five serotypes included in this vaccine and that were traditionally associated with resistance (6B, 9V, 14, 19F and 23F), still accounted for a significant fraction of isolates resistant to either erythromycin, penicillin or both (45%), and this proportion declined only slightly from what was seen in 2006–2008 (47%) [6]. Both penicillin and erythromycin non-susceptibility, that is concentrated in the serotypes included in PCVs, has risen in adult IPD. The emergence of multiresistant serotype 19A isolates in the post-PCV7 period in adult IPD played a major role in preventing the decline of resistance in IPD in Portugal by compensating the decline of resistant isolates expressing PCV7 serotypes. The significant decrease of erythromycin resistance noted in 2011 may signal a change in this trend, but there were multiple serotypes responsible for this decline and no significant concentration in PCV13 serotypes was noted.

In contrast to our expectations, the use of PCV7 and now of PCV13 has not resulted in further declines of the PCV7 serotypes as causes of adult IPD. The continued importance of these serotypes is in contrast to the massive declines that lead to the almost elimination of PCV7 serotypes as causes of adult IPD in the USA [9,16]. The reasons behind this difference are possibly multifactorial and may include: 1) differences in the transmission dynamics of these serotypes in the USA and Portugal; 2) differences in the clonal composition or in the selective pressures exerted upon the pneumococcal populations; 3) a relatively slow uptake of PCV7 in Portugal when compared to the USA; and 4) a lower coverage of PCV7 vaccination in children in Portugal. Although we cannot formally exclude the first two possibilities, we believe that their impact would be transient since the pneumococcal population would adapt to these new circumstances. On the other hand, the later two possibilities are particularly interesting and suggest that in order to obtain the full public health benefits of vaccinating children with PCV13, one should aim at the rapid introduction of the vaccine and at vaccination coverage higher than the 75% currently achieved in Portugal. The proportion of PCV7 serotypes has shown little change since 2005, when the proportion of PCV7 serotypes causing IPD in adults declined from pre-PCV7 levels to its current values. It is therefore likely that the stability of PCV7 serotypes in the intervening six years corresponds to a new steady-state related to the lower vaccination coverage of children in Portugal relative to that in the USA or England and Wales [12,19].

The recent replacement with the 13-valent formulation for the vaccination of children in Portugal, as has happened in many countries, may equally lead to herd effects in the additional serotypes included in this vaccine. The approval for the use of PCV13 in adults raises the possibility that adult vaccination may not only reduce IPD due to vaccine serotypes in vaccinees but also lead to reduced carriage of the serotypes included in the vaccine, as was seen in children, further compounding the herd effect noted from childhood vaccination. However, even without adult vaccination, continued use of conjugate vaccines in children and the herd effects they produce together with sustained high antimicrobial usage, are likely to drive alterations in the serotypes causing adult IPD that will influence the fraction of potentially vaccine preventable disease in this age group.

Our study was not designed to allow the estimate of the incidence of IPD and it therefore does not evaluate potential changes in incidence with time and in particular since PCV7 introduction. However, the design based on the reporting of all laboratory confirmed IPD cases with isolation of the etiological agent within the surveillance network, the large number of isolates studied, the wide coverage of the country by the network and the stable number of isolates reported in each year, guarantees that the data accurately represents IPD in Portugal and can be used to evaluate changes in the relative importance of the different serotypes. PPV23 availability since 1996 had only minor effects on the proportion of adult IPD caused by PPV23 serotypes but due to its limited use in the country [6] maybe none should be expected. The proportion of infections potentially covered by PPV23 in Portugal remained always above 80% since surveillance was started in 1999, except in 2011 when it dropped slightly below that level (Fig. 1). This occurred despite significant serotype changes in this period. In contrast, the fraction of adult IPD potentially preventable by PCV13 that had increased diminished in recent years reaching 53.5% in 2011 and may be even lower in patients with co-morbidities [41]. PCV13 use could still potentially prevent more than half of adult IPD in Portugal at the time it was licensed for use in adults >50 yrs. The dynamics of the serotypes causing IPD in adults justify the continued surveillance of these infections in order to evaluate the potential coverage afforded by each of the two currently available vaccines with an adult indication.

Supporting Information

Figure S1 Proportion of penicillin non-susceptible pneumococci (PNSP) and erythromycin resistant pneumococci (ERP) (1999–2011). The period 1999–2003, previously identified as the pre-PCV7 period, was analyzed together. (PDF)

Table S1 Number of isolates expressing serotypes included in the 13-valent conjugate vaccine but not included in the 7-valent conjugate vaccine causing invasive infections in Portugal (2008–2011).
(PDF)

Table S2 Capsular types of the isolates recovered from CSF between 2009 and 2011.
(PDF)

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Author Contributions

Conceived and designed the experiments: MR JM-C. Performed the experiments: ANH JD-M SIA. Analyzed the data: ANH MR JM-C. Contributed reagents/materials/analysis tools: PGSSI. Wrote the paper: ANH MR JM-C.

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Serotype 3 Remains the Leading Cause of Invasive Pneumococcal Disease in Adults in Portugal (2012–2014) Despite Continued Reductions in Other 13-Valent Conjugate Vaccine Serotypes

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Since 2010 the 13-valent pneumococcal conjugate vaccine (PCV13) replaced the 7-valent vaccine (PCV7) as the leading pneumococcal vaccine used in children through the private sector. Although, neither of the PCVs were used significantly in adults, changes in adult invasive pneumococcal disease (IPD) were expected due to herd protection. We characterized $n = 1163$ isolates recovered from IPD in adults in 2012–2014 with the goal of documenting possible changes in serotype prevalence and antimicrobial resistance. Among the 54 different serotypes detected, the most frequent, accounting for half of all IPD, were serotypes: 3 (14%), 8 (11%), 19A (7%), 22F (7%), 14 (6%), and 7F (5%). The proportion of IPD caused by PCV7 serotypes remained stable during the study period (14%), but was smaller than in the previous period (19% in 2009–2011, $p = 0.003$). The proportion of IPD caused by PCV13 serotypes decreased from 51% in 2012 to 38% in 2014 ($p < 0.001$), mainly due to decreases in serotypes 7F and 19A. However, PCV13 serotype 3 remained relatively stable and the most frequent cause of adult IPD. Non-PCV13 serotypes continued the increase initiated in the late post-PCV7 period, with serotypes 8 and 22F being the most important emerging serotypes. Serotype 15A increased in 2012–2014 (0.7% to 3.5%, $p = 0.011$) and was strongly associated with antimicrobial resistance. However, the decreases in resistant isolates among serotypes 14 and 19A led to an overall decrease in penicillin non-susceptibility (from 17 to 13%, $p = 0.174$) and erythromycin resistance (from 19 to 13%, $p = 0.034$). Introduction of PCV13 in the NIP for children, as well as its availability for adults may further alter the serotypes causing IPD in adults in Portugal and lead to changes in the proportion of resistant isolates.

Keywords: ***Streptococcus pneumoniae*, conjugate vaccines, polysaccharide vaccine, antimicrobial resistance, invasive disease, serotype**

INTRODUCTION

Two types of pneumococcal vaccines are licensed to prevent invasive pneumococcal disease (IPD), both targeting a restricted number of serotypes out of the 94 serotypes currently recognized in *Streptococcus pneumoniae*: strictly polysaccharide based vaccines and polysaccharide-protein conjugate based vaccines (PCVs) (Ramirez, 2014). The first licensed pneumococcal conjugate vaccine was the 7-valent pneumococcal conjugate vaccine (PCV7), which targets serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. PCV7 became available for children in the USA in 2000 and in Europe in 2001. Two additional conjugate vaccines became available more recently: a 10-valent vaccine (PCV10), which includes PCV7 serotypes and serotypes 1, 5, and 7F; and a 13-valent vaccine (PCV13), which includes PCV10 serotypes and serotypes 3, 6A, and 19A. PCVs proved to be highly effective in reducing the number of IPD episodes caused by vaccine serotypes (Pilishvili et al., 2010; Aguiar et al., 2014). Moreover, a decrease in IPD caused by PCV serotypes was also noted in non-vaccinated individuals (a phenomenon termed herd protection) (Horácio et al., 2013; Moore et al., 2015). However, use of PCVs was also accompanied by replacement of vaccine serotypes by non-vaccine types (NVTs) as causes of IPD, both in vaccinated children and in non-vaccinated adults. The overall impact of this phenomenon varied greatly around the world (Pérez-Trallero et al., 2009; Guevara et al., 2014; Harboe et al., 2014; Moore et al., 2015; Waight et al., 2015). The switch to the higher valency vaccines PCV10 and PCV13 also affected emerging serotypes. For instance, serotypes 7F and 19A were reported as emerging in IPD in the post-PCV7 period (Aguiar et al., 2010; Steens et al., 2013; Guevara et al., 2014; Harboe et al., 2014; Waight et al., 2015) but several studies have already shown that they decrease following PCV13 use (Aguiar et al., 2014; Moore et al., 2015; Waight et al., 2015). A 23-valent strictly polysaccharide vaccine (PPV23) includes 12 of the serotypes found in PCV13 (except 6A) and serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F. This vaccine has been used for two decades in older children and adults and has proven efficacy in the prevention of IPD (Moberley et al., 2013).

PCV7, PCV10 and PCV13 became available in Portugal in late-2001, mid-2009 and in early-2010, respectively. However, in contrast to many European countries, in Portugal PCV7 was not included in the national immunization program (NIP) and the uptake of PCV7 in children increased gradually over time, reaching 75% in 2008 (Aguiar et al., 2008). PCV13 replaced PCV7 since its availability and has been the most widely used pneumococcal vaccine since then, with estimates of 63% coverage in 2012 (Aguiar et al., 2014). PCV13 received an indication for adults ≥50 years in 2012 and in 2013 its indication was extended to all ages, but use of these vaccines in adults in Portugal was believed to be low until 2014. PCV13 was introduced into the NIP for children in 2015, being given free of charge to all children born from January 2015 onwards, with a 2+1 schedule (Direcção Geral de Saúde, 2015b). PPV23 is also available in Portugal since 1996, but its uptake among adults is estimated to be low (∼10%) (Horácio et al., 2012). Since 2015, guidelines from the national health authorities recommend vaccinating adults in particular risk groups with both PCV13 and PPV23 (Direcção Geral de Saúde, 2015a). However, these groups will constitute a minority of the overall population and there are no guidelines recommending vaccinating adults more broadly with any of the pneumococcal vaccines.

In spite of the gradual increase in PCV uptake in children and the relatively modest coverage, we found significant changes in serotype distribution and antimicrobial susceptibility of pneumococci causing adult IPD that could be attributed at least in part to herd protection. The proportion of adult IPD caused by PCV13 serotypes was highest in 2008 (70%), but a gradual decrease took place until 2011, when only 54% of the isolates causing adult IPD expressed PCV13 serotypes (Horácio et al., 2012, 2013). In the present study we continued monitoring potential changes in serotype distribution and antimicrobial susceptibility of isolates causing adult IPD after PCV13 received an adult indication and before the introduction of PCV13 in the NIP for children.

MATERIALS AND METHODS

Ethics Statement

Case reporting and isolate collection were considered to be surveillance activities and were exempt from evaluation by the Review Board of the Faculdade de Medicina of Universidade de Lisboa. The data and isolates were de-identified so that these were irretrievably unlinked to an identifiable person.

Bacterial Isolates

Invasive pneumococcal infections have been monitored in Portugal since 1999 by the Portuguese Group for the Study of Streptococcal Infections (Serrano et al., 2004). This is a laboratory-based surveillance system, in which 31 microbiology laboratories throughout Portugal are asked to identify all isolates responsible for IPD and to send them to a central laboratory for characterization. Although, the laboratories were contacted periodically to submit the isolates to the central laboratory, no audit was performed to ensure compliance, which may be variable in this type of study. A case of IPD was defined by the isolation of pneumococci from a normally sterile fluid, such as blood, pleural fluid or cerebral spinal fluid (CSF). The isolates included in the study were recovered from adult patients (≥18 years) with IPD between January 2012 and December 2014. Only one isolate from each patient in each year was included in the study. All isolates were identified as pneumococci by colony morphology, hemolysis on blood agar plates, optochin susceptibility and bile solubility.

Serotyping and Antimicrobial Susceptibility Testing

Serotypes were determined by the standard capsular reaction test using the chessboard system and specific sera (Sørensen, 1993) (Statens Serum Institut, Copenhagen, Denmark). Serotypes were classified into vaccine serotypes, i.e., those included in PCV7 (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F), in PCV10 (all PCV7 serotypes plus serotypes 1, 5, and 7F), in PCV13 (all PCV10 serotypes plus 3, 6A, and 19A) or in PPV23 (all

PCV13 serotypes, except serotype 6A and serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F) and non-vaccine serotypes (NVT). Given the high frequency of spontaneous switching between serotypes 15B and 15C we have opted to group isolates with these serotypes into a single group. Due to difficulties in phenotypically distinguishing isolates of serotype 25A and serogroup 38 these were also grouped together into the 25A/38.

Minimal inhibitory concentrations (MICs) for penicillin and cefotaxime were determined using Etest strips (Biomérieux, Marcy l'Étoile, France). In 2008, the CLSI changed the recommended breakpoints used to interpret MIC values. Unless otherwise stated we have used the CLSI-recommended breakpoints prior to 2008 (Clinical and Laboratory Standards Institute, 2007) as epidemiological breakpoints that allow the comparison with previous studies. Isolates were further characterized by determining their susceptibility to erythromycin, clindamycin, vancomycin, linezolid, tetracycline, levofloxacin, trimethroprim-sulfamethoxazole and chloramphenicol by the Kirby-Bauer disk diffusion technique, according to the CLSI recommendations and interpretative criteria (Clinical and Laboratory Standards Institute, 2014).

Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin, as previously described (Melo-Cristino et al., 2003). Simultaneous resistance to erythromycin and clindamycin defines the MLS_B phenotype (resistance to macrolides, lincosamides and streptogramin B) while non-susceptibility only to erythromycin indicates the M phenotype.

Statistical Analysis

Simpson's index of diversity (SID) and respective 95% confidence intervals (CI95%) was used to measure the population diversity (Carriço et al., 2006). Adjusted Wallace (AW) coefficients were used to compare two sets of partitions (Severiano et al., 2011). These indices were calculated using the online tool available at http://www.comparingpartitions.info. Differences were evaluated by the Fisher exact test and the Cochran-Armitage test (CA) was used for trends with the false discovery rate (FDR) correction for multiple testing (Benjamini and Hochberg, 1995). A $p < 0.05$ was considered significant for all tests.

RESULTS

Isolate Collection

A total of 1163 isolates were collected from adults with invasive pneumococcal disease between 2012 and 2014: 404 in 2012, 383 in 2013 and 376 in 2014. The majority were recovered from blood ($n = 1066$, 91.7%) and the remaining from CSF ($n = 59$, 5.1%), pleural fluid ($n = 26$, 2.2%), peritoneal fluid ($n = 9$, 0.8%) and other normally sterile sites ($n = 3$, 0.3%).

Serotype Distribution

Between 2012 and 2014, a total of 54 different serotypes were identified. The most frequent, which accounted for half of the isolates were serotypes 3 ($n = 161$, 13.8%), 8 ($n = 123$, 10.6%), 19A ($n = 84$, 7.2%), 22F ($n = 79$, 6.8%), 14 ($n = 73$, 6.3%), and 7F ($n = 61$, 5.2%). **Figures 1–3** represent the number of isolates expressing serotypes included in PCVs, the additional serotypes found in PPV23, and the number of isolates expressing NVTs stratified by age group. Serotype diversity was high (2012–2014 SID = 0.944, CI95%: 0.939–0.949). Although, diversity was >0.93 in all the studied years, there was a small but significant increase in serotype diversity between 2012 (SID = 0.935, CI95%: 0.924–0.945) and 2013 (SID = 0.950, CI95%: 0.942–0.958) ($p = 0.019$).

Serotype distribution varied according to age group but serotype diversity was not different in the three age groups considered (18–49 years, SID = 0.948, CI95%: 0.938–0.958; 50–64 years, SID = 0.945, CI95%: 0.933–0.957; ≥65 years, SID = 0.939, CI95%: 0.931–0.946). Only for serotype 1 were the differences in age distribution statistically supported after FDR correction with the proportion of serotype 1 decreasing with age (accounting for 6.5, 2.6, and 0.6% of the isolates recovered from patients aged 18–49 years, 50–64 years and ≥65 years, respectively, CA $p < 0.001$). In contrast, the proportion of IPD caused by the group of additional serotypes found only in PCV13 (3, 6A, and 19A) increases with age (15.2% in 18–49 years, 19.9% in 50–64 years and 24.7% in ≥65 years, CA $p = 0.002$, significant after FDR).

When considering serotypes presenting three or more CSF isolates, we found a positive association with CSF for serotypes 19F ($p = 0.006$) and 23B ($p = 0.005$), both significant after FDR correction (Table S1). No significant associations with serotype were found for isolates recovered from pleural fluid.

Figure 4 shows the proportion of potentially vaccine preventable IPD during the study period and, for comparison purposes, also from 2008 to 2011 since important changes in serotype distribution initiated in this period (Horácio et al., 2013). Considering the current study period only (2012–2014), the overall proportion of IPD caused by PCV7 serotypes remained stable, while there was a decrease in the proportion of IPD caused by the additional serotypes found in both PCV10 and PCV13 (serotypes 1, 5, 7F; from 11.1 to 4.8%, $p = 0.001$, significant after FDR) and in PCV13 only (serotypes 3, 6A, and 19A; from 26.5 to 19.9%, $p = 0.024$, significant after FDR). This resulted in the overall decrease in the proportion of IPD caused by PCV13 serotypes from 51.2% in 2012 to 38.0% in 2014 ($p < 0.001$, significant after FDR). The proportion of IPD caused by PPV23 serotypes and NVTs did not suffer significant changes during the study period (**Figure 4**). However, the proportion of IPD caused by the additional serotypes found only in PPV23 (PPV23 add) significantly increased, from 27.2 to 38.0% ($p = 0.001$, significant after FDR). When considering the evolution of potentially vaccine preventable IPD in the entire period from 2008 to 2014, there was a decrease in the overall proportion of IPD caused by PCV13 serotypes, although this was temporarily interrupted in 2012, mainly due to a slight increase of serotype 3 (see below).

Table 1 shows the evolution of individual serotypes causing adult IPD from 2008 to 2014. When looking for trends in the proportion of individual serotypes during the current study period (2012–2014), the only significant change that was supported after FDR correction was the decrease in serotype

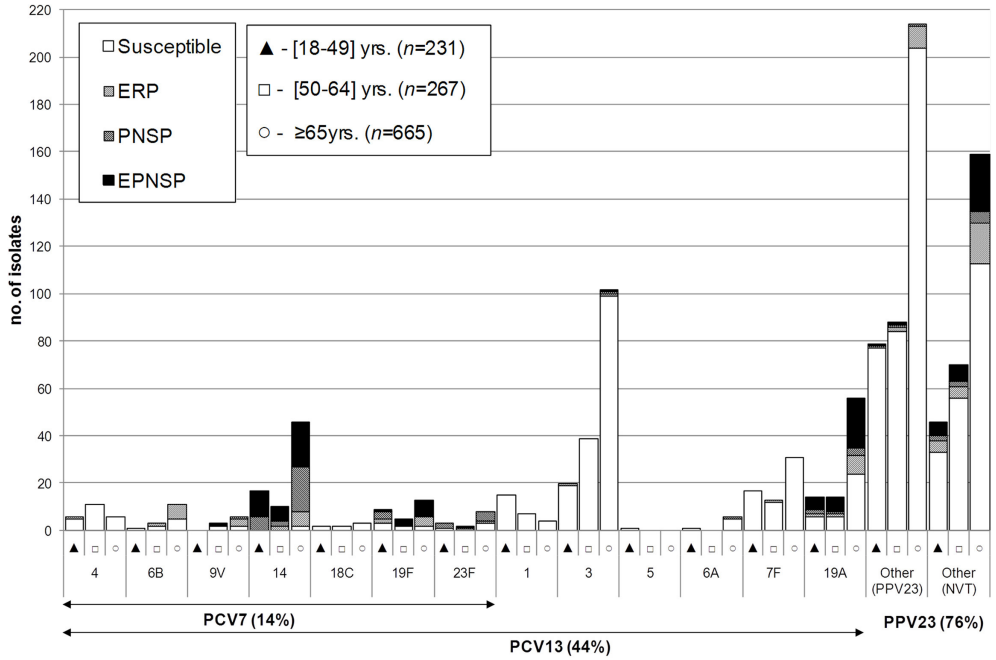

FIGURE 1 | Serotypes of isolates causing invasive pneumococcal disease in adult patients (≥18 years) in Portugal, 2012–2014. The number of isolates expressing each serotype in each of the age groups considered is indicated. Isolates recovered from patients 18–49 years are indicated by black triangles, from patients 50–64 years by open squares, and from patients ≥65 years by open circles. Isolates presenting both erythromycin resistance and penicillin non-susceptibility (EPNSP) are represented by black bars. Penicillin non-susceptible isolates (PNSP) are indicated by dark hatched bars. Erythromycin resistant pneumococci (ERP) are indicated by light hatched bars. Isolates susceptible to both penicillin and erythromycin are represented by white bars. The serotypes included in the seven-valent conjugate vaccine (PCV7) and in the 13-valent conjugate vaccine (PCV13) are indicated by the arrows. NVT, non-vaccine serotypes; PPV23, 23-valent polysaccharide vaccine.

7F (from 8.2% in 2012 to 4.7% in 2013 and 2.7% in 2014, CA $p < 0.001$). No significant changes in the proportion of individual serotypes were detected during the study period when stratifying by age group (data not shown).

When considering together data from 2008 to 2014 there were changes (significant after FDR) in the proportion of individual serotypes. There were decreases in the proportion of IPD caused by serotypes: 1 (from 13.4 to 1.9%, CA $p < 0.001$), 5 (from 2.9 to 0.3%, CA $p < 0.001$), 9V (from 3.4 to 0.3%, CA $p < 0.001$) and 19A (from 11.7 to 5.6%, CA $p = 0.005$). In contrast, there were increases in the proportion of IPD caused by PPV23 serotypes: 8 (from 3.7 to 12.2%, CA $p < 0.001$), 22F (from 2.4 to 8.2%, CA $p < 0.001$) and 20 (from 1.0 to 3.7%, CA $p = 0.001$); and an increase of the NVT 15A (from 1.0 to 3.5%, CA $p = 0.002$). Even though these changes were statistically supported when analyzing data from 2008 to 2014, in the case of serotypes 19A and 15A, the more disparate values were only detected from 2013 onwards, while for serotype 20, this occurred from 2012 onwards.

Table 2 shows the evolution of IPD serotypes during the study period (2012–2014) according to vaccine serotypes and stratified by age group. Recapitulating what was seen when considering all age groups together (**Figure 4**), a decrease in the overall proportion of IPD caused by PCV13 serotypes was detected in the three age groups considered; however, only for individuals ≥65 years was this statistically supported (**Table 2**). Moreover, only for this age group was the decrease in the additional serotypes found in both PCV10 and PCV13 (serotypes 1, 5 and 7F) statistically supported after FDR correction (**Table 2**). When analyzing the evolution of each serotype from 2008 to 2014 stratifying by age group, only serotype 1 decreased in all age groups considered (CA $p < 0.001$ for each, significant after FDR correction), while the increase of serotype 8 was significant only in the two older groups (≥50 years) (CA $p < 0.001$ for both, significant after FDR correction), and the changes in serotypes 5, 7F, 19A, 20, and 22F were statistically supported only in individuals ≥65 years (CA $p < 0.001$ for serotypes 5 and 7F, CA $p = 0.007$ for serotype 19A, CA $p = 0.003$ for serotype 20 and CA $p = 0.001$ for serotype 22F, all significant after FDR correction).

Antimicrobial Susceptibility

Resistance to the antimicrobials tested is summarized in **Table 3**. A total of $n = 179$ isolates (15.4%) were classified as penicillin non-susceptible pneumococci (PNSP): $n = 160$ (89.4%) presenting low level resistance and $n = 19$ (10.6%), high level resistance. Considering current CLSI breakpoints for penicillin, $n = 12/59$ CSF isolates (20.3%) would have been considered resistant and only $n = 5/1104$ non-CSF isolates (0.5%) would have been considered intermediately resistant. A total of $n = 198$ isolates (17.0%) were classified as erythromycin resistant pneumococci (ERP). Of these, $n = 159$ presented the MLS_B phenotype, while the remaining ($n = 39$, 19.7%) presented the M

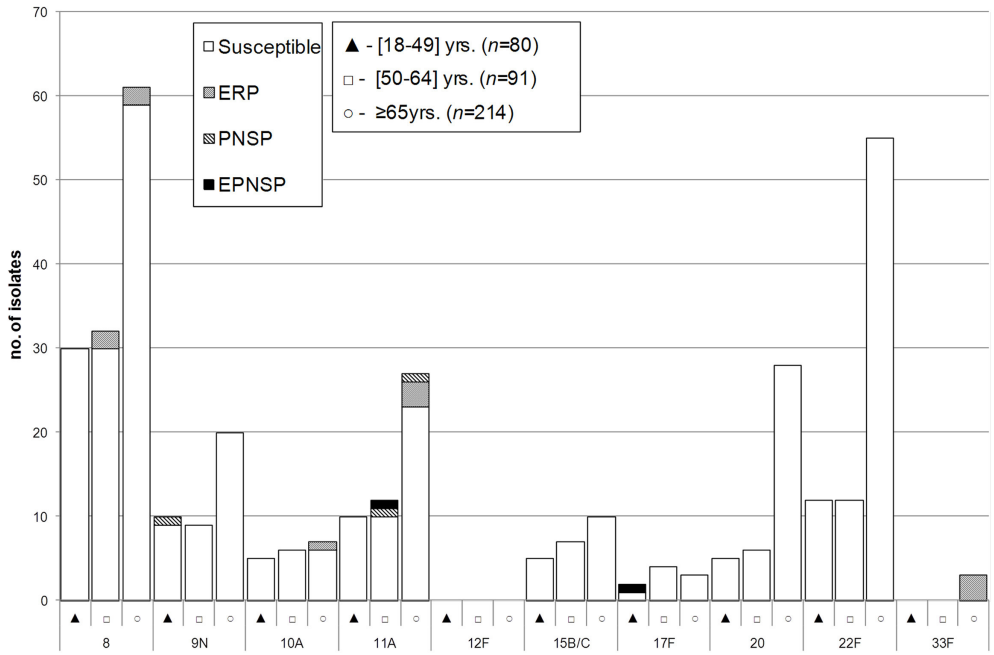

FIGURE 2 | Isolates expressing serotypes present in PPV23 but not included in conjugate vaccines causing invasive pneumococcal disease in adult patients (≥18 years) in Portugal, 2012–2014. See legend of **Figure 1**. Out of the 11 serotypes present in PPV23 but absent from PCV13, serotype 2 was not found in our collection.

phenotype. Isolates simultaneously non-susceptible to penicillin and erythromycin (EPNSP) accounted for 10.4% of the collection ($n = 121$).

Antimicrobial resistance did not change significantly between age groups. In 2012–2014, there was a significant decrease in antimicrobial resistance for several antimicrobials—erythromycin resistance decreased from 18.8 to 13.0% (CA $p = 0.034$), clindamycin resistance decreased from 16.1 to 10.4% (CA $p = 0.022$) and tetracycline resistance decreased from 13.4 to 7.7% (CA $p = 0.010$). Although, not statistically supported, there was also a decrease in penicillin non-susceptibility, from 16.8% in 2012 to 13.3% in 2014 (CA $p = 0.174$).

There was some correlation between serotype and antimicrobial resistance (**Figures 1**–**3**). The AW for serotype and PNSP was 0.569 (CI95%: 0.507–0.631) and the AW for serotype and ERP was 0.527 (CI95%: 0.458–0.596). Serotypes 14 and 19A were the most frequent serotypes among PNSP and ERP. Serotype 14 accounted for 35.2% of PNSP and 22.2% of ERP while serotype 19A occurred in 21.2% of PNSP and 21.2% of ERP. Taken together, PCV7 serotypes accounted for 48.6% of PNSP, 37.9% of ERP and 40.5% of EPNSP. Considering the PCV13 serotypes, these constituted 71.5, 61.1, and 67.8% of PNSP, ERP and EPNSP, respectively. The additional serotypes found in PPV23 but not in PCV13 accounted for only 2.8, 6.6, and 1.7% of PNSP, ERP and EPNSP, respectively. The proportion of resistant isolates was higher among isolates expressing NVTs: 25.7, 32.3, and 30.6% of PNSP, ERP and EPNSP, respectively (**Figures 1**–**3**). The most frequent NVTs among PNSP and ERP were serotypes 6C and 15A, which together accounted for 19.0% of PNSP and 18.2% of ERP (**Figure 3**).

DISCUSSION

The decrease in PCV13 serotypes observed previously (Horácio et al., 2012, 2013) continued during the present study period resulting in only 38.0% of the isolates collected in 2014 expressing PCV13 serotypes (**Figure 4**). However, different serotypes underlie the changes in 2008–2011 and 2012–2014.

The timeframes of the decreases seen for serotypes 7F and 19A are consistent with a possible herd protection of childhood vaccination with the most recently introduced PCVs. Similar decreases in serotypes 7F and 19A as causes of adult IPD followed the use of PCV13 in children in the USA (Moore et al., 2015) and in several European countries (Steens et al., 2013; Guevara et al., 2014; Harboe et al., 2014; Waight et al., 2015). Decreases in the incidence of IPD caused by these two serotypes were also documented among children in Portugal (Aguiar et al., 2014). In Portugal the decrease in serotype 7F preceded that of serotype 19A in adult IPD. This could have been attributed to the use of PCV10 in children, since PCV10 includes serotype 7F but not serotype 19A. Moreover, this vaccine was introduced in Portugal months earlier than PCV13. However, in children, serotype 19A decreased as a cause of IPD before an effect of PCV13 was expected and before any decrease in serotype 7F (Aguiar et al., 2014). This points to the importance of other factors besides vaccination in triggering

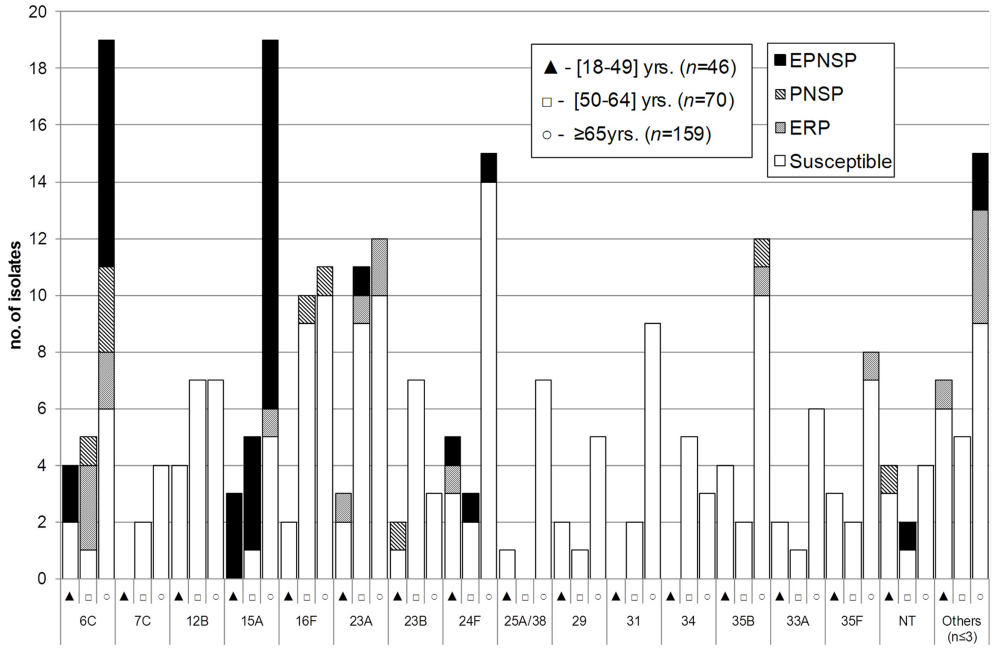

FIGURE 3 | Isolates expressing serotypes not included in any pneumococcal vaccine causing invasive pneumococcal disease in adult patients (≥18 years) in Portugal, 2012–2014. See legend of **Figure 1**. NT, non-typable. Isolates expressing serotype 25A and 38 could not be distinguished phenotypically and are represented together. Only serotypes including $n > 3$ isolates are discriminated.

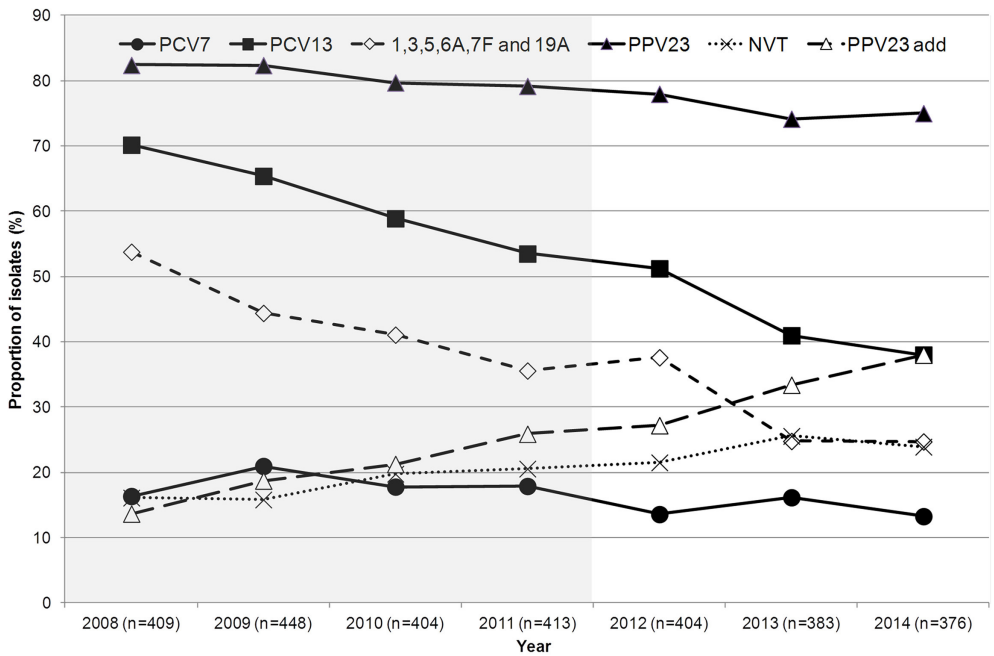

FIGURE 4 | Proportion of isolates expressing serotypes included in pneumococcal vaccines causing invasive pneumococcal disease in adult patients (≥18 years) in Portugal, 2008–2014. The data up to 2011 were presented previously (Horácio et al., 2012, 2013).

changes in serotype prevalence and suggest that the initial changes seen in serotype 7F IPD in adults are the result of secular trends.

In contrast to these serotypes, there was no overall reduction of serotype 3. These results are concordant with other studies that failed to show a consistent reduction of serotype 3 among adult

TABLE 1 | Serotypes of the isolates responsible for invasive pneumococcal disease in adult patients (≥18 years), 2008–2014.

Serotype	No. of isolates (%)							CA[a]	CA
					Current study period				
	2008	2009	2010	2011	2012	2013	2014	2012–2014	2008–2014
PCV13									
1	55 (13.4)	48 (10.7)	22 (5.4)	17 (4.1)	12 (3.0)	7 (1.8)	7 (1.9)	0.289	**<0.001**
3	51 (12.5)	53 (11.8)	59 (14.6)	48 (11.6)	66 (16.3)	45 (11.7)	50 (13.3)	0.209	0.613
4	10 (2.4)	12 (2.7)	17 (4.2)	14 (3.4)	6 (1.5)	8 (2.1)	9 (2.4)	0.361	0.352
5	12 (2.9)	9 (2.0)	4 (1.0)	0 (0)	0 (0)	0 (0)	1 (0.3)	0.211	**<0.001**
6A	6 (1.5)	8 (1.8)	2 (0.5)	1 (0.2)	2 (0.5)	1 (0.3)	4 (1.1)	0.315	0.062
6B	1 (0.2)	7 (1.6)	3 (0.7)	9 (2.2)	5 (1.2)	5 (1.3)	5 (1.3)	0.909	0.272
7F	48 (11.7)	48 (10.7)	35 (8.7)	43 (10.4)	33 (8.2)	18 (4.7)	10 (2.7)	**0.001**	**<0.001**
9V	14 (3.4)	7 (1.6)	8 (2.0)	5 (1.2)	4 (1.0)	4 (1.0)	1 (0.3)	0.255	**<0.001**
14	29 (7.1)	45 (10.0)	30 (7.4)	31 (7.5)	29 (7.2)	26 (6.8)	18 (4.8)	0.172	0.045
18C	0 (0)	6 (1.3)	1 (0.2)	1 (0.2)	1 (0.2)	4 (1.0)	2 (0.5)	0.588	0.675
19A	48 (11.7)	33 (7.4)	44 (10.9)	38 (9.2)	39 (9.7)	24 (6.3)	21 (5.6)	0.027	0.005
19F	7 (1.7)	13 (2.9)	8 (2.0)	5 (1.2)	9 (2.2)	12 (3.1)	6 (1.6)	0.576	0.956
23F	6 (1.5)	4 (0.9)	5 (1.2)	9 (2.2)	1 (0.2)	3 (0.8)	9 (2.4)	0.005	0.618
PPV23 add									
8	15 (3.7)	19 (4.2)	27 (6.7)	33 (8.0)	34 (8.4)	43 (11.2)	46 (12.2)	0.081	**<0.001**
9N	10 (2.4)	12 (2.7)	13 (3.2)	11 (2.7)	8 (2.0)	13 (3.4)	18 (4.8)	0.030	0.122
10A	3 (0.7)	8 (1.8)	7 (1.7)	6 (1.5)	2 (0.5)	8 (2.1)	8 (2.1)	0.062	0.294
11A	7 (1.7)	13 (2.9)	10 (2.5)	16 (3.9)	16 (4.0)	18 (4.7)	15 (4.0)	0.974	0.012
12F	0 (0)	1 (0.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	–	0.334
15B/C	8 (2.0)	4 (0.9)	3 (0.7)	8 (1.9)	5 (1.2)	9 (2.3)	8 (2.1)	0.353	0.096
17F	3 (0.7)	2 (0.4)	4 (1.0)	4 (1.0)	5 (1.2)	2 (0.5)	2 (0.5)	0.255	0.981
20	4 (1.0)	8 (1.8)	5 (1.2)	7 (1.7)	14 (3.5)	11 (2.9)	14 (3.7)	0.851	**0.001**
22F	10 (2.4)	17 (3.8)	16 (4.0)	22 (5.3)	25 (6.2)	23 (6.0)	31 (8.2)	0.261	**<0.001**
33F	0 (0)	0 (0)	1 (0.2)	0 (0)	1 (0.2)	1 (0.3)	1 (0.3)	0.959	0.180
NVT[b]									
6C	4 (1.0)	13 (2.9)	13 (3.2)	10 (2.4)	8 (2.0)	14 (3.7)	6 (1.6)	0.757	0.600
15A	4 (1.0)	5 (1.1)	5 (1.2)	5 (1.2)	3 (0.7)	11 (2.9)	13 (3.5)	0.011	**0.002**
23A	6 (1.5)	8 (1.8)	8 (2.0)	4 (1.0)	9 (2.2)	8 (2.1)	9 (2.4)	0.879	0.317
16F	3 (0.7)	8 (1.8)	3 (0.7)	7 (1.7)	13 (3.2)	3 (0.8)	7 (1.9)	0.161	0.233
24F	3 (0.7)	6 (1.3)	5 (1.2)	2 (0.5)	5 (1.2)	9 (2.3)	9 (2.4)	0.241	0.027
12B	10 (2.4)	5 (1.1)	3 (0.7)	11 (2.7)	6 (1.5)	8 (2.1)	4 (1.1)	0.649	0.700
35B	2 (0.5)	5 (1.1)	10 (2.5)	5 (1.2)	6 (1.5)	4 (1.0)	8 (2.1)	0.480	0.222
35F	2 (0.5)	3 (0.7)	2 (0.5)	3 (0.7)	7 (1.7)	4 (1.0)	2 (0.5)	0.110	0.350
23B	4 (1.0)	3 (0.7)	7 (1.7)	6 (1.5)	4 (1.0)	5 (1.3)	3 (0.8)	0.801	0.958
31	2 (0.5)	2 (0.4)	1 (0.2)	5 (1.2)	5 (1.2)	2 (0.5)	4 (1.6)	0.786	0.197
NT	0 (0)	3 (0.7)	4 (1.0)	3 (0.7)	1 (0.2)	3 (0.8)	6 (1.6)	0.042	0.060
33A	1 (0.2)	4 (0.9)	8 (2.0)	4 (1.0)	2 (0.5)	5 (1.3)	2 (0.5)	0.929	0.913
25A/38	2 (0.5)	3 (0.7)	0 (0)	2 (0.5)	3 (0.7)	3 (0.8)	2 (0.5)	0.726	0.583
29	0 (0)	0 (0)	0 (0)	0 (0)	4 (1.0)	2 (0.5)	2 (0.5)	0.433	0.009
34	2 (0.5)	0 (0)	0 (0)	8 (1.9)	3 (0.7)	1 (0.3)	4 (1.1)	0.605	0.138
7C	2 (0.5)	2 (0.4)	2 (0.5)	1 (0.2)	1 (0.2)	4 (1.0)	1 (0.3)	0.942	0.883
18A	6 (1.5)	0 (0)	1 (0.2)	2 (0.5)	0 (0)	3 (0.8)	0 (0)	0.959	0.080
21	3 (0.7)	0 (0)	0 (0)	4 (1.0)	0 (0)	0 (0)	0 (0)	–	0.108
Others[c]	8 (2.0)	1 (0.2)	8 (2.0)	3 (0.7)	7 (1.7)	9 (2.3)	8 (2.1)	–	–
Total	409	448	404	413	404	383	376	–	–

[a] *CA, Cochran Armitage test of trend. In bold are the serotypes with significant p-values ($p < 0.05$) after FDR correction.*

[b] *NVT, non-vaccine serotypes.*

[c] *Only serotypes detected in ≥3 isolates in at least one year are shown; the remaining are represented in "Others."*

TABLE 2 | Number of isolates responsible for invasive pneumococcal disease in adult patients (≥18 years), according to vaccine serotype groups and age groups, 2012–2014.

	Serotype groups	No. isolates (%)			CA[a]
		2012	2013	2014	
18–49 years	PCV7[b]	18 (21.4)	12 (15.0)	8 (11.9)	0.112
	1, 5, and 7F	15 (17.9)	10 (12.5)	8 (11.9)	0.286
	3, 6A, and 19A	12 (14.3)	12 (15.0)	11 (16.4)	0.719
	PCV13[c]	45 (53.6)	34 (42.5)	27 (40.3)	0.094
	PPV23 add[d]	26 (31.0)	29 (36.3)	24 (35.8)	0.511
	NVTs[e]	13 (15.5)	17 (21.3)	16 (23.9)	0.191
50–64 years	PCV7[b]	7 (9.2)	14 (13.9)	15 (16.7)	0.164
	1, 5, and 7F	10 (13.2)	6 (5.9)	4 (4.4)	0.037
	3, 6A, and 19A	20 (26.3)	19 (18.8)	14 (15.6)	0.087
	PCV13[c]	37 (48.7)	39 (38.6)	33 (36.7)	0.124
	PPV23 add[d]	17 (22.4)	34 (33.7)	37 (41.1)	0.011
	NVTs[e]	22 (28.9)	28 (27.7)	20 (22.2)	0.316
≥65 years	PCV7[b]	30 (12.3)	36 (17.8)	27 (12.3)	0.947
	1, 5, and 7F	20 (8.2)	9 (4.5)	6 (2.7)	**0.008**
	3, 6A, and 19A	75 (30.7)	39 (19.3)	50 (22.8)	0.042
	PCV13[c]	125 (51.2)	84 (41.6)	83 (37.9)	0.004
	PPV23 add[d]	67 (27.5)	65 (32.2)	82 (37.4)	0.022
	NVTs[e]	52 (21.3)	53 (26.2)	54 (24.7)	0.384

[a]CA, Cochran Armitage test of trend. In bold are the serotype groups with significant p-values ($p < 0.05$) after FDR correction.
[b]PCV7, serotypes included in the 7-valent pneumococcal conjugate vaccine.
[c]PCV13, serotypes included in the 13-valent pneumococcal conjugate vaccine.
[d]PPV23 add, the additional 11 serotypes present in the 23-valent pneumococcal polysaccharide vaccine but absent from the 13-valent pneumococcal conjugate vaccine.
[e]NVTs, serotypes not included in any of the currently available pneumococcal vaccines.

TABLE 3 | Antimicrobial resistance of the isolates responsible for invasive pneumococcal disease in adult patients (≥18 years) in Portugal, 2012–2014.

	No. resistant isolates (%)		
	18–49 years ($n = 231$)	50–64 years ($n = 267$)	≥65 years ($n = 665$)
PEN	40 (17.3)	32 (12.0)	107 (16.1)
MIC_{90}	0.38	0.125	0.25
MIC_{50}	0.016	0.012	0.016
CTX	4 (1.7)	3 (1.1)	6 (0.9)
MIC_{90}	0.25	0.19	0.25
MIC_{50}	0.016	0.016	0.016
LEV	0 (0)	1 (0.4)	6 (0.9)
ERY	34 (14.7)	37 (13.9)	127 (19.1)
CLI	31 (13.4)	30 (11.2)	100 (15.0)
CHL	5 (2.2)	6 (2.2)	8 (1.2)
SXT	30 (13.0)	39 (14.6)	93 (14.0)
TET	23 (10.0)	21 (7.9)	79 (11.9)

PEN, penicillin; CTX, cefotaxime; LEV, levofloxacin; ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; SXT, trimethoprim/sulphamethoxazole; TET, tetracycline. All isolates were susceptible to vancomycin and linezolid.

IPD after the use of PCV13 in children (Steens et al., 2013; Harboe et al., 2014; Moore et al., 2015; Waight et al., 2015) and with a study that demonstrated a low and non-significant effectiveness of PCV13 against serotype 3 IPD in children (Andrews et al., 2014).

The proposed higher efficacy of PCV13 against serotype 19F (Dagan et al., 2013) cannot explain the decrease in proportion of PCV7 serotypes, since serotype 19F was uncommon in our collection and no significant decrease was seen between the two periods (**Table 1**). The reduction of the overall proportion of IPD caused by PCV7 serotypes was instead related with decreases in serotypes 4, 9V and 14 (**Table 1**). Among these, serotype 4 exhibited the most significant decrease. Since the most significant decrease of serotype 14 IPD was detected in 2014, it remains uncertain if it will be sustained in the following years. Serotype 14 has been the most frequent PCV7 serotype causing adult IPD in Portugal, both before and after PCV7 use in children. This could be associated with particular characteristics of the highly successful and resistant clone Spain^{14}-ST156, to which this serotype was found to be associated (Horácio et al., 2016). High antimicrobial consumption in our country could also contribute significantly to maintain resistant clones such as this one in circulation.

The non-PCV serotypes that increased the most since the late-post PCV7 period were those found in PPV23, especially serotypes 8, 22F, and 20 (ranked by frequency); but also the non-PPV23 serotype 15A (**Table 1**). Serotypes 15A and 22F were found in carriage in adults in Portugal (Almeida et al., 2014), while serotypes 8 and 20 were not found in carriage in adults and were shown to have a high invasive disease potential (Sá-Leão et al., 2011). Serotype 8 was the second most frequent cause of IPD during the current study period and in 2013 and 2014 was the most frequent cause of IPD among younger adults (18–49 years). Serotype 8 increased in importance as a cause of IPD in other countries, being the most frequent cause of IPD in patients aged >5 years in England and Wales after the introduction of PCV13 (Waight et al., 2015) and also important in adult IPD elsewhere (Guevara et al., 2014; Regev-Yochay et al., 2015). Serotype 22F became the second most frequent cause of IPD in adults aged ≥65 years in 2013 and 2014. In the USA, this serotype was the most common cause of adult IPD in the post-PCV13 period (Moore et al., 2015). An increase of serotype 22F after PCV13 use was also reported in Canada (Demczuk et al., 2013) and in some European countries (Steens et al., 2013; Lepoutre et al., 2015). Serotype 20 increased more modestly and only among individuals aged ≥65 years. An increase of this serotype was also noted in Canada, although mostly among individuals aged 15–49 years (Demczuk et al., 2013). Taken together, these observations indicate that, although there may be some regional differences, there are serotypes that seem to be consistently emerging in different geographic locations in the post-PCV13 period. These may reflect circulating serotypes in asymptomatic carriers but also serotypes with an enhanced invasive disease potential.

In 2014, the last year of the study, serotype 15A surpassed serotype 19A and 14 to become the most frequent serotype

among ERP and was the second most frequent serotype among PNSP behind serotype 14. The overall decreases observed in PNSP and ERP were not only due to decreases in the total number of isolates expressing serotypes 14 and 19A, which were not compensated by the increase in serotype 15A (**Table 1**), but also to an unexpected decrease in the proportion of resistant isolates within serotypes 14 and 19A. While 72% of serotype 14 and 64% of serotype 19A were ERP in 2012, only 44% of serotype 14 and 33% of serotype 19A were ERP in 2014 ($p = 0.071$ and $p = 0.031$, respectively). Similarly, there was a decrease in the proportion of PNSP among serotype 19A, from 59% in 2012 to 24% in 2014 ($p = 0.014$).

Our surveillance system is exclusively laboratory based and lacks compliance audits, so our study was not designed to estimate the incidence of adult IPD. However, we did note a slight decrease in the number of isolates sent to us in 2013 and 2014 (**Figure 4**). This could reflect a net reduction of adult IPD following PCV13 use in children, as reported by others (Guevara et al., 2014; Harboe et al., 2014; Lepoutre et al., 2015; Moore et al., 2015; Regev-Yochay et al., 2015) and seen with IPD in children in Portugal (Aguiar et al., 2014). Alternatively, this could reflect lower reporting by participating laboratories. We also noted a marked decrease in the number of isolates recovered from younger patients relative to either of the older age groups when comparing 2009–2011 to 2012–2014 ($p < 0.001$) (**Figure 1**) (Horácio et al., 2013). Even if the decrease in number of isolates is attributed to lower reporting, we have no reason to believe that this would affect preferentially a particular age group. We also have no indication of changes in clinical practice (such as blood culturing practices), which could influence these results. We therefore believe that the most likely explanation is a true reduction in incidence of IPD in 18–49 years old individuals, in agreement with a study from the UK that found that this group was the one where the decrease in IPD incidence was more pronounced and followed more closely PCV13 use in children (Waight et al., 2015).

As discussed above, our study was not designed to allow the estimate of the incidence of IPD and it therefore does not evaluate potential changes in incidence with time. Specifically, although we include the majority of medical centers in Portugal our surveillance is not comprehensive and we did not perform audits to ensure that participating centers reported all cases, namely we did not include cases for which no viable pneumococcal isolate was received for characterization. However, the design based on the reporting of all isolates causing IPD within the surveillance network, the large number of isolates studied, the wide coverage of the country by the network and the stable number of reporting centers, guarantees that the data accurately represents IPD in Portugal and can be used to evaluate changes in the relative importance of the different serotypes.

In spite of relatively modest vaccine coverage (63% in 2012), there were major changes in the serotype distribution of the pneumococcal population responsible for adult IPD in Portugal following the use of PCVs in children consistent with herd protection. These changes have contributed also to significant reductions in antimicrobial resistance. The recent inclusion of PCV13 in the NIP for children in Portugal may have an even greater impact on IPD in adults. This remarkable effect of PCVs in protecting non-vaccinated individuals may question the need of using PCV13 directly in vaccinating adults. Still, data from 2014 indicates that the overall proportion of adult IPD caused by PCV13 serotypes remained significant (38%) and that isolates expressing PPV23 serotypes accounted for 75% of all IPD. Taken together this suggests a key role of vaccination in any effective management strategy of IPD.

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AUTHOR CONTRIBUTIONS

JM and MR: Conceived and designed the experiments. PGSSI: Collected data. AH, CS, and JL: Performed the experiments. AH, JM, and MR: Analyzed the data. All authors contributed to the writing of the manuscript and approved the version to be submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01616

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The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RESEARCH ARTICLE

Population Structure of *Streptococcus pneumoniae* Causing Invasive Disease in Adults in Portugal before PCV13 Availability for Adults: 2008-2011

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Abstract

Among the 1660 isolates recovered from invasive pneumococcal disease (IPD) in adults (> = 18 yrs) in 2008–2011, a random sample of ≥50% of each serotype (n = 871) was chosen for MLST analysis and evaluation for the presence and type of pilus islands (PIs). The genetic diversity was high with 206 different sequence types (STs) detected, but it varied significantly between serotypes. The different STs represented 80 clonal complexes (CCs) according to goeBURST with the six more frequent accounting for more than half (50.6%) of the isolates—CC156 (serotypes 14, 9V and 23F), CC191 (serotype 7F), CC180 (serotype 3), CC306 (serotype 1), CC62 (serotypes 8 and 11A) and CC230 (serotype 19A). Most of the isolates (n = 587, 67.3%) were related to 29 Pneumococcal Molecular Epidemiology Network recognized clones. The overall proportion of isolates positive for any of the PIs was small (31.9%) and declined gradually during the study period (26.6% in 2011), mostly due to the significant decline of serotype 1 which is associated with PI-2. The changes in serotypes that occurred in adult IPD after the introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) for children were mostly due to the expansion of previously circulating clones, while capsular switching was infrequent and not related to vaccine use. The reduction of IPD caused by PCV7 serotypes in the years following PCV7 implementation did not result in a decline of antimicrobial resistance in part due to the selection of resistant genotypes among serotypes 14 and 19A.

data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction

The 7-valent conjugate vaccine (PCV7) was available for children through the private sector in Portugal from 2001 onwards until it was replaced in the beginning of 2010 by the 13-valent conjugate vaccine (PCV13). In 2012, PCV13 received approval for use also in adults > 50 years of age with an extension being made to all ages in 2013. Additionally, PCV13 entered the Portuguese National Immunization Program (NIP) in June 2015 for children born from January 2015 onwards. Two other vaccines, the 23-valent pneumococcal polysaccharide vaccine (PPV23) and the 10-valent conjugate vaccine (PCV10), have also been available in Portugal since 1996 and 2009, respectively, but with a low uptake [1].

Among the more than 90 different pneumococcal serotypes identified, only a few cause the majority of IPD. While for some serotypes the capsular polysaccharide is the dominant determinant of invasiveness, for others distinct genotypes show important differences in invasiveness [2]. Additionally, there are other features that are strongly associated with genotype independently of serotype, such as antimicrobial susceptibility and the presence and type of pilus islands [3,4]. With the availability of pneumococcal conjugate vaccines that efficiently target particular serotypes, important changes have been reported regarding not only serotype but also genotype distributions of pneumococci causing IPD [5–9]. Interestingly, while non-vaccine serotypes have emerged as a cause of IPD, in some cases distinct clones expressing the same serotype have risen in frequency in different geographic regions [10,11].

While numerous studies have addressed the serotype distribution of IPD, information regarding the clonal composition of pneumococcal populations has been scarcer. In a previous study we defined the clonal composition of pneumococci causing IPD in both children and adults in the pre-PCV7 period [11]. In a subsequent study we documented major changes in the potential coverage of PCV13 starting in 2009, due to decreases in prevalence of serotypes 1 and 5 [12]. In the present study we aimed to characterize the clonal composition of pneumococci causing adult IPD in Portugal between 2008 and 2011, a period characterized by extensive use of PCV7 and the adoption of PCV13 in children and prior to the use of PCV13 in adults.

Materials and Methods

Bacterial isolates

The isolates included in this study were recovered from adult patients (≥18 yrs) with invasive pneumococcal disease between 2008 and 2011 and were characterized in previous studies regarding serotype distribution and antimicrobial susceptibility [1,12]. A case of invasive disease was defined by the recovery of pneumococci from a normally sterile source, such as blood or cerebral spinal fluid (CSF). Serotypes were grouped into conjugate vaccine serotypes, i.e., those included in PCV13 (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 19A, 23F) that comprise all serotypes found in lower valency vaccines (PCV7: 4, 6B, 9V, 14, 18C, 19F, 23F; and PCV10: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F), those included in PPV23 (all serotypes included in PCV13 except 6A and serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F), and non-vaccine serotypes (NVT). The isolates that were not typable with any of the complete set of sera available from the Staten Serum Institute (Copenhagen, Denmark) were considered non-typable (NT). Given the high frequency of spontaneous switching between serotypes 15B and 15C we opted to include strains with these serotypes into a single group. Due to the difficulty in distinguishing a set of isolates that were positive for both serotypes 25A and 38 we opted to include strains with these serotypes into a single group.

From a total of 1660 isolates recovered, a random sample of ≥50% of the isolates (n = 871) from each serotype and from each year was chosen to be characterized by MLST and tested for

the presence of the pilus islands. Briefly, among the 1660 isolates, there were 52 different serotypes, with the 10 most frequent being serotypes 3 (13.0%), 7F (10.0%), 19A (9.8%), 1 (8.5%), 14 (8.1%), 8 (5.7%), 22F (3.9%), 4 (3.2%), 9N (2.8%) and 11A (2.8%). However, the 10 most frequent serotypes were different in each of the age groups. In the 18–49 yr olds (n = 472) these were serotypes 1 (14.0%), 7F (11%), 8 (9.1%), 14 (8.7%), 3 (7.6%), 19A (6.1%), 9N (4.2%), 4 (3.8%), 22F (3.2%), 11A (2.8%). In the 50–64 yr olds (n = 358) these were serotypes 3 (12%), 19A (10.3%), 1 (9.5%), 7F (9.2%), 14 (6.1%), 4 (5.0%), 8 (4.5%), 11A (3.4%), 22F (3.4%), 9V (2.8%). In the ≥65 yr olds (n = 830) these were serotypes 3 (15.8%), 19A (11.6%), 7F (10.2%), 14 (8.7%), 1 (4.9%), 22F (4.6%), 8 (4.2%), 6C (3.0%), 11A (2.5%), 9N (2.3%). Overall, the proportions of PCV7, PCV13 and PPV23 serotypes were 18.4%, 61.9% and 79.4%, respectively. Non-susceptibility to penicillin, defined as either intermediate level penicillin resistance (MIC 0.12–1.0 μg/ml) or high level resistance (MIC≥2.0 μg/ml) as discussed previously [1,12], was found in 330 isolates (19.9%), while 315 isolates (19.0%) were resistant to erythromycin. The age and sex of the patients and the source of the isolates randomly chosen for further study was similar to that of the 1660 isolates. In the genotyped group the age distribution was as follows: 28.5% of the isolates were from individuals 18–49 yrs, 21.2% from 50–64 yrs and 50.3% from ≥65 yrs. The majority of the isolates were collected from blood (87.9%), 8.4% from CSF, 2.5% from pleural fluid and 1.2% from other normally sterile sources.

MLST

MLST was performed as described previously [13]. The DNA sequences were analyzed using Bionumerics software (Applied-Maths, Sint-Martens-Laten, Belgium) and the alleles and sequence types were assigned according to the pneumococcal MLST database available at http://pubmlst.org/spneumoniae/. The goeBURST algorithm [14] implemented in the PHYLOViZ software [15] was used to establish relationships between STs. Clonal complexes were defined at the single-locus-variant (SLV) and double-locus-variant (DLV) levels.

Detection of Pilus Islands

The presence of pilus islets (PI) was evaluated by PCR. Briefly, for PI-1 in the absence of the pilus islet, a product of 1-3Kb was expected using primers PFL-up and P-dn flanking the islet [4]. In strains yielding no PCR product, the *rlrA* gene was detected using primers RLRA-up and RLRA-dn. A similar approach was followed to detect the presence of PI-2 [16].

Statistical Analysis

Sample diversity was evaluated using the Simpson's index of diversity (SID) and the respective 95% confidence intervals (CI95%) [17]. To compare two sets of partitions the Adjusted Wallace (AW) coefficients were calculated [18] using the online tool available at http://www.comparingpartitions.info. Differences were evaluated by the Fisher exact test with the false discovery rate (FDR) correction for multiple testing [19] and the Cochran-Armitage test was used for trends. A $p<0.05$ was considered significant for all tests.

Results

Sequence Type Distribution and Relationship with Serotype

The 871 isolates analyzed by MLST presented 206 different STs (SID = 0.971, CI95%: 0.967–0.976) grouping into 80 CCs (SID = 0.948, CI95%: 0.942–0.953) according to goeBURST analysis, when including all STs deposited in the database. The 14 most frequent STs, which accounted for more than half of the genotyped isolates (50.6%) were, in decreasing order,

ST191 (9.9%), ST306 (7.0%), ST180 (6.9%), ST53 (4.5%), ST156 (4.0%), ST276 (3.6%), ST433 (3.2%), ST66 (2.8%), ST408 (1.7%), ST232 (1.7%), ST260 (1.5%), ST143 (1.4%), ST179 (1.3%) and ST289 (1.3%).

Twenty new allelic combinations and 19 new alleles were identified. The new allelic combinations were identified as STs: 6176, 6177, 6180, 6181, 6182, 6973, 8866, 9955, 9956, 9957, 9958, 9960, 9963, 9966, 9969, 9970, 9971, 9979, 9982 and 9986. The novel alleles identified were designated 200, 307 and 309 for *aroE*, 429, 636 and 637 for *ddl*, 294, 295, 428 and 430 for *gdh*, 437 and 438 for *gki*, 273 for *recP* and 588, 589, 590, 592, 593 and 605 for *xpt*.

There was a strong correlation between CC and the vaccine serotype groups (AW = 0.810, CI95%: 0.763–0.857), with the six most prevalent CCs being mainly composed of isolates presenting vaccine serotypes (95.5%). Table 1 shows the age distribution and serotypes of the most frequent STs found in the 22 major CCs (n≥10 isolates), together accounting for 83.7% of the genotyped isolates. The major CC (CC156, n = 101) included mostly isolates expressing PCV7 serotypes, namely 14, 9V and 23F, while four of the remaining five most frequent CCs were mainly composed of isolates presenting the additional serotypes found in PCV13 (mainly 7F, 3, 1 and 19A). The other most frequent lineage, CC62, consisted mostly of isolates expressing serotypes included only in PPV23 (serotypes 8 and 11A). The age distribution and serotypes of the STs found in CCs with <10 isolates are shown in S1 Table.

Fig 1 shows the STs expressing each of the 13 serotypes included in PCV13 and Fig 2 the STs expressing each of the 10 most frequent serotypes found among those not included in any of the conjugate vaccines. The STs found in the remaining serotypes are indicated in S2 Table. The genetic diversity varied greatly with serotype, with serotypes, 4, 6A, 6B, 9V, 18C, 19A, 20 and 23A being highly diverse (SID>0.8) and serotypes 1, 5, 7F, 9N and 22F displaying very limited diversity (SID<0.3). In general, there was a predominance of high genetic diversity among PCV13 serotypes and low genetic diversity among the 10 most frequent non-PCV13 serotypes. For serotypes 9V, 14 and 23A, the wide variety of STs did not result in a high diversity of CCs, with a maximum of two CCs being detected in each. The genetic diversity of each serotype was independent of the serotype's frequency. Examples of this are the low frequency serotypes 6B and 18C that presented a high genetic diversity and no dominant ST.

A total of 587 isolates (67.3%) presented STs related to 29 of the 43 clones recognized by the Pneumococcal Molecular Epidemiology Network (PMEN) [20], sharing at least five MLST alleles with these clones (357 isolates had the same ST, 133 were SLVs and 97 were DLVs). When considering these isolates the predominant clones were Netherlands^{7F}-191 (n = 88), Spain^{9V}-156 (n = 71), Netherlands^{3}-180 (n = 68), Netherlands^{8}-53 (n = 63), Sweden^{1}-306 (n = 63), Denmark^{14}-230 (n = 47), Tennessee^{14}-67 (n = 24), Tennessee^{23F}-37 (n = 24) and Netherlands^{15B}-199 (n = 21) (Figs 1 and 2 and S2 Table). Additionally, another 63 isolates were included in the same CCs of other four PMEN clones.

The correlation between ST and serotype was high (AW = 0.942, CI95%: 0.912–0.973), but there were STs that presented more than one serotype (Table 1 and S1 and S2 Tables). The serotype distribution along the studied years for the STs expressing more than one serotype is shown in Table 2.

Variation of STs with Time

When analyzing the evolution of STs between 2008 and 2011 we identified some fluctuations, although the majority reflected changes in serotype prevalence occurring in this period. However, while for ST306 (serotype 1) there was a decline, significant after correcting for multiple testing (from 11.0% to 2.8%, Cochran-Armitage test of trend p = 0.014), for the other STs the changes were only significant before FDR correction. The STs for which there was a significant

Table 1. Age distribution and the serotypes of the most frequent STs found in the 22 major CCs (n≥10 isolates) identified by goeBURST.

			no. of isolates per age group				
CC (n)	ST	Total	[18–49]	[50–64]	> = 65	Dominant serotype (n)	Other serotypes
156 (101)	156	35	13	5	17	14 (31)	9V (3), 10A (1)
	143	12	2	2	8	14 (12)	-
	338	10	3	1	6	23F (7)	23A (2), 19F (1)
	162	6	2	1	3	9V (4)	19F (1), 24A (1)
	2944	5	0	3	2	14 (5)	-
	Others[a]	33	8	6	19	9V (10)	14 (8), 6B (5), 6C (3), 23F (3), 35F (2), 17F (1), 17A (1)
191 (88)	191	86	31	15	40	7F (83)	7A (1), NT[b] (2)
	Others[a]	2	0	2	0	7F (2)	-
180 (68)	180	60	8	17	35	3 (60)	-
	Others[a]	8	2	1	5	3 (8)	-
306 (68)	306	61	26	14	21	1 (61)	-
	350	5	1	2	2	1(5)	-
	Others[a]	2	2	0	0	1(2)	-
62 (67)	53	39	15	8	16	8 (37)	NT[b] (2)
	408	15	6	4	5	11A (14)	11C (1)
	62	7	1	2	4	11A (7)	-
	Others[a]	6	3	0	3	8 (2), 11A (2)	18C (1), 22F (1)
230 (47)	276	31	3	7	21	19A (31)	-
	230	6	1	1	4	24F (4)	19A (2)
	Others[a]	10	4	3	3	19A (8)	10A (1), 24F (1)
81 (30)	66	24	12	3	9	9N (23)	NT[b] (1)
	Others[a]	6	1	0	5	24F (4)	4 (2)
433 (29)	433	28	4	5	19	22F (28)	-
	Others[a]	1	0	0	1	22F (1)	-
439 (25)	439	7	0	3	4	23B (7)	-
	42	5	1	3	1	23A (4)	6A (1)
	Others[a]	13	3	2	8	23A (7)	23B (3), 23F (3)
15 (24)	9	8	3	0	5	14 (8)	
	1201	7	3	0	4	19A (4)	7C (3)
	Others[a]	9	1	2	6	14 (5)	34 (2), 6B (1), 7C (1)
177 (24)	179	11	4	4	3	19F (11)	-
	Others[a]	13	3	5	5	19A (5)	19F (3), 21 (3), 15A (1), 15 B/C (1)
199 (21)	416	8	1	2	5	19A (8)	-
	411	7	1	2	4	15B/C (7)	-
	199	6	2	2	2	19A (3)	15B/C (2), 18C (1)
378 (19)	232	15	3	5	7	3 (15)	-
	Others[a]	4	2	0	2	3 (4)	-
113 (16)	123	5	1	0	4	17F (5)	-
	1766	5	2	0	3	31 (5)	-
	Others[a]	6	1	2	3	22F (3)	17F (1), 18C (1), 31 (1)
460 (16)	97	10	2	3	5	10A (10)	-
	Others[a]	6	3	1	2	6A (4)	10A (1), 35F (1)
260 (15)	260	13	2	3	8	3 (13)	-
	Others[a]	2	1	0	1	3 (2)	-
218 (13)	218	10	3	2	5	12B (10)	-
	Others[a]	3	1	1	1	12B (2)	12F (1)

(*Continued*)

Table 1. (*Continued*)

			no. of isolates per age group				
CC (n)	**ST**	**Total**	**[18–49]**	**[50–64]**	**> = 65**	**Dominant serotype (n)**	**Other serotypes**
289 (13)	289	11	5	2	4	5 (11)	-
	Others[a]	2	1	0	1	5 (2)	-
30 (11)	30	10	2	2	6	16F (10)	-
	Others[a]	1	0	0	1	16F (1)	-
63 (11)	63	8	3	0	5	15A (7)	15F (1)
	Others[a]	3	0	1	2	3 (1), 7F (1), 15A (1)	-
315 (11)	386	7	1	1	5	6C (6)	6B (1)
	Others[a]	4	0	1	3	6C (3)	6B (1)
404 (10)	404	9	5	0	4	8 (9)	-
	Others[a]	1	1	0	0	8 (1)	-

[a] Sequence types that accounted for less than 5 isolates each were grouped together in "Others".

[b] NT-non typable.

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p-value in the Cochran-Armitage test for trends but unsupported after FDR correction were: ST53 (serotype 8), that increased from 3.3% to 6.5% (p = 0.043); ST289 (serotype 5), that accounted for 2.4% of IPD in 2008 and 0% in 2011 (p = 0.020); ST717 (serotypes 33A and 33F) that increased from 0% to 1.4% (p = 0.048); and STs 193 (serotype 19A) and 409 (serotype 14) that were only detected in 2008 (1.9% and 1.4%, respectively; p = 0.001 and p = 0.020, respectively). Regarding changes in CCs with time, these reflected the changes identified in STs, with only CC306 declining significantly after FDR correction (from 12.9% to 2.8%, Cochran-Armitage test of trend p = 0.001).

Relationship of STs with Patient Age and Isolate Source

When grouping the isolates according to the three patient age groups– 18–49 yrs, 50–64 yrs and > = 65 yrs—only CC5902 showed a statistically significant association with age. The seven isolates belonging to this CC were all recovered from individuals with 18–49 yrs (p = 0.011, significant after FDR correction, S1 Table).

When testing for associations between STs and CCs and isolate source, the only significant association found was between CC460 and CSF, with 6 out of 16 isolates being collected from CSF (p = 0.012, significant after FDR correction).

Presence of Pilus Islands

A total of 278 isolates, representing 31.9% of the genotyped collection, carried at least one PI. Among these, 107 (38.5%) had only PI-1, 165 (59.4%) only PI-2 and 6 (2.2%) presented the two PIs simultaneously.

While the proportion of PI-1 positive isolates remained stable between 2008 and 2011 (from 10.0% to 11.6%, Cochran-Armitage test of trend p = 0.857), there was a significant decline of PI-2 carrying isolates (from 24.8% to 15.8%, Cochran-Armitage test of trend p = 0.007). This also resulted in an overall increase in the proportion of isolates lacking any of the pilus islands, from 63.8% in 2008 to 72.6% in 2011 (p = 0.013).

The presence and variants of the PIs were more strongly associated with ST (AW = 0.950, CI95%: 0.933–0.967) than with serotype (AW = 0.711, CI95%: 0.651–0.771). The STs that were

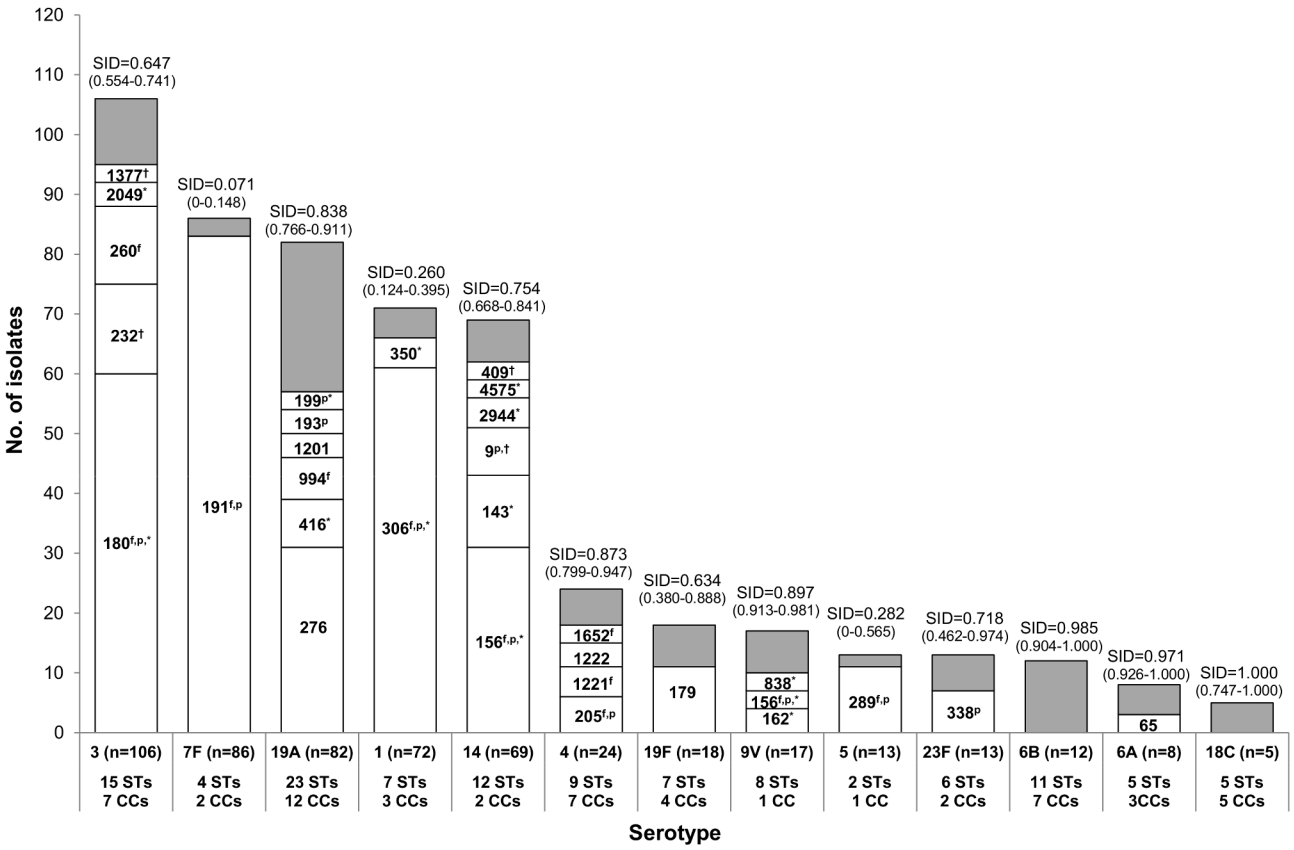

Fig 1. Distribution of STs according to serotype of the isolates causing adult IPD in 2008–2011 and expressing serotypes included in the conjugate vaccines. The STs that were considered by goeBURST as founders of a CC are indicated by "^{f}". The STs that matched the STs of PMEN clones are indicated by "^{P}". Marked either with "*" or "†" are STs belonging to the same CC in each serotype. The respective SID values are indicated on top of the bars and in parenthesis are the respective confidence intervals. In grey are represented the isolates included in STs with <3 isolates. These were: **serotype 4** –ST801 (n = 2) and STs 244, 246, 259 and 1866 (n = 1, each); **serotype 6B** – ST176 (n = 2), STs 138, 273, 386, 473, 1518, 6175, 9957, 9970, 9986 and 10051 (n = 1, each); **serotype 9V** –STs 280 and 10044 (n = 2, each) and STs 239, 1762 and 10054 (n = 1, each); **serotype 14** –ST15 (n = 2) and STs 2511, 2616, 4573, 4576 and 10041 (n = 1, each); **serotype 18C** –STs 102, 113, 199, 1233 and 10033 (n = 1, each); **serotype 19F** –ST177 (n = 2), STs 89, 162, 271, 338 and 391 (n = 1, each); **serotype 23F** –ST10039 (n = 2) and STs 1135 and 9579 (n = 1, each); **serotype 1** –STs 217, 228, 1233, 3081 and 4578 (n = 1, each); **serotype 3** –ST1220 (n = 2) and STs 505, 1230, 6014, 9162 and 10038 (n = 1, each); **serotype 5** –STs 280 and 10044 (n = 2, each), STs 239, 1762 and 10054 (n = 1, each); **serotype 6A** –ST1876 (n = 2) and STs 42, 460 and 10055 (n = 1, each); **serotype 7F** –STs 1062, 1589 and 3130 (n = 1, each) and **serotype 19A** –STs 230, 242, 320, 2013 and 6174 (n = 2, each) and STs 241, 878, 2102, 2669, 2732, 4197, 4847, 6178, 6973, 9963 and 10042 (n = 1, each).

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significantly associated with PI-1 and PI-2 are shown in Table 3. All isolates included in CC320 (n = 3) and CC2669 (n = 3) presented the two PIs simultaneously.

Among the 105 isolates presenting only PI-1, 87.9% expressed PCV7 serotypes, namely serotypes 14 (n = 49), 4 (n = 15), 19F (n = 13), 9V (n = 11) and 6B (n = 6). The remaining isolates were from serotypes 19A (n = 9) and 7F, 24A and 35B (n = 1, each). PI-2 positive isolates were from serotypes 7F (n = 85), 1 (n = 68), 11A (5), 19A (n = 2), 3, 7A and 31 (n = 1, each) and NT (n = 2). The isolates presenting simultaneously the two types of PIs were from serotypes 19A (n = 5) and 19F (n = 1).

No associations between isolate source and type of PI were detected. Still, there was a low proportion of PI-2 positive isolates among isolates recovered from the CSF, with only 6 of the 73 CSF isolates presenting PI-2, and while 7 of the 15 isolates recovered from pleural fluid carried PI-2, none carried PI-1.

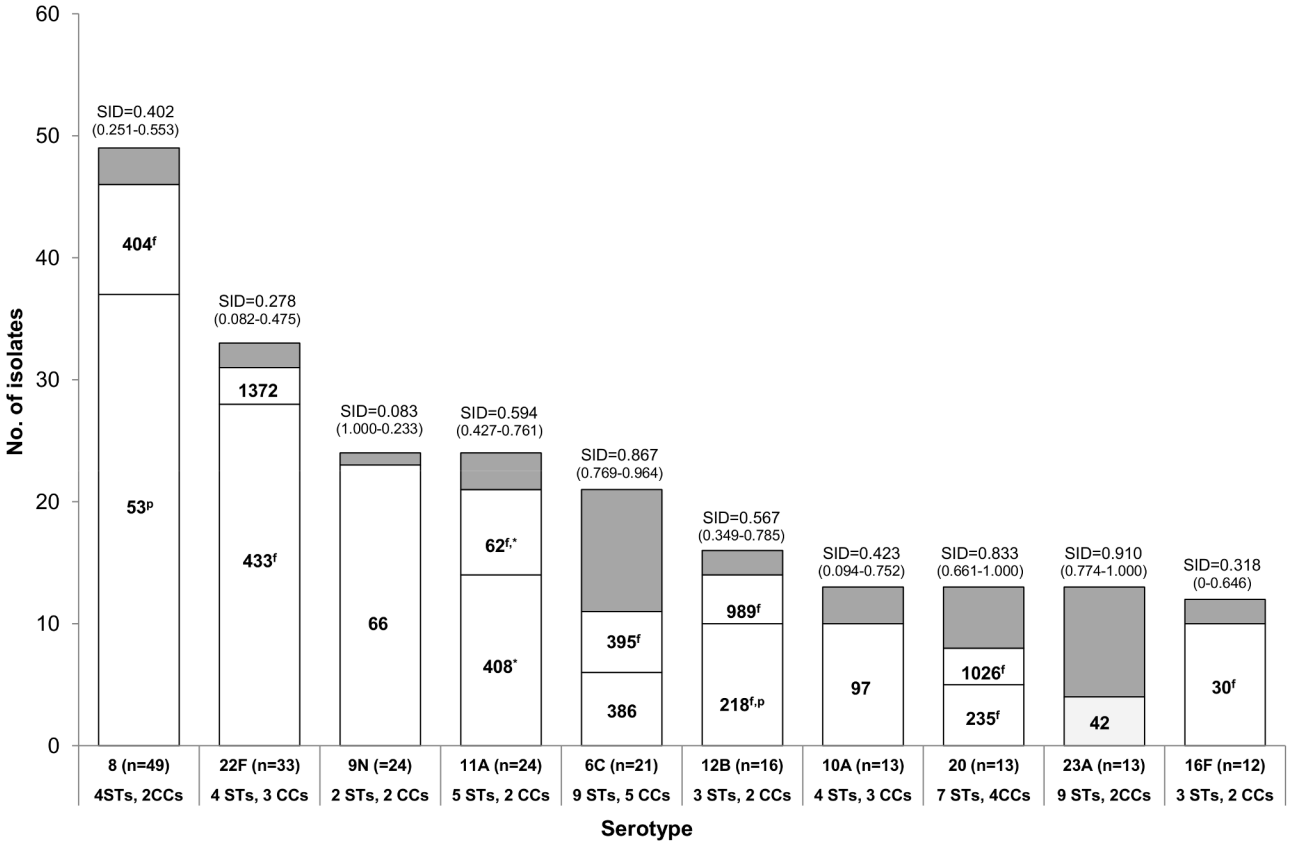

Fig 2. Distribution of STs according to serotype of the isolates causing adult IPD in 2008–2011 and expressing the 10 most frequent serotypes not included in any of the conjugate vaccines. The respective SID values are shown on top of the bars and in parenthesis are the respective confidence intervals. In grey are represented the isolates included in STs with <3 isolates. These were: **serotype 8** –ST1012 (n = 2) and ST 9969 (n = 1); **serotype 22F** –STs 10053 and 10220 (n = 1, each); **serotype 9N** –ST3982 (n = 1); **serotype 11A** –STs 9955, 9960 and 10052 (n = 1, each); **serotype 6C** –STs 1150, 1692 and 3396 (n = 2, each) and STs 1390, 1715, 2667 and 4310 (n = 1, each); **serotype 12B** –ST6180 (n = 2); **serotype 10A** –STs 156, 816 and 3135 (n = 1, each); **serotype 20** –STs 1483, 1871, 7221, 9958 and 10047 (n = 1, each); **serotype 23A** –ST338 (n = 2) and STs 190, 311, 438, 6177, 7960, 8866 and 10048 (n = 1, each); **serotype 16F** –STs 570 and 5902 (n = 1, each).

doi:10.1371/journal.pone.0153602.g002

Antimicrobial Resistance

Similarly to pilus islands, resistance to antimicrobials was more strongly associated with ST than with serotype. The AW for ST or serotype and penicillin susceptibility was, respectively, 0.785 (CI95%: 0.729–0.841) and 0.389 (CI95%: 0.326–0.452), while the AW for ST or serotype and erythromycin susceptibility was, respectively, 0.711 (CI95%: 0.598–0.824) and 0.315 (CI95%:0.217–0.413). The sequence types that were associated with penicillin non-susceptible pneumococci (PNSP) and erythromycin resistant pneumococci (ERP) are presented in Table 4.

Discussion

In spite of several years of PCV7 use in children, the most frequent CC was CC156 (11.6%, Table 1), a lineage that expressed mainly PCV7 serotypes (89.1%) and which was also the most frequent in IPD in the pre-PCV7 period [11]. We had previously shown that the serotype distribution of pneumococci causing adult IPD had changed significantly in the post-PCV7 period, with the proportion of PCV7 serotypes declining to values below 20% [1,12,21]. Adult

Table 2. Serotype distribution for the STs expressing more than one serotype between 2008–2011.

	Serotype (n)			
ST[a] (n)	2008	2009	2010	2011
156 (35)	14 (6), 9V (1)	14 (12), 9V (1)	14 (7), 10A (1)	14 (6), 9V (1)
338 (10)	-	23F (3)	23F (2), 23A (1), 19F (1)	23F (2), 23A (1)
717 (9)	-	33A (1)	33A (4), 33F (1)	33A (2), 3 (1)
63 (8)	15A (2)	15A (3)	15A (2), 15F (1)	-
386 (7)	-	6C (2)	6C (2)	6C (2), 6B (1)
1201 (7)	19A (2), 7C (1)	19A (1), 7C (1)	19A (1), 7C (1)	-
162 (6)	9V (3), 19F (1)	-	9V (1)	24A (1)
199 (6)	15B/C (1)	15B/C (1), 19A (2), 18C (1)	-	19A (1)
230 (6)	24F (2)	-	24F (2), 19A (1)	19A (1)
42 (5)	6A (1)	23A (2)	23A (2)	-
241 (5)	18A (3)	-	18A (1), 19A (1)	-

[a] Only the sequence types that presented $\geq$ 5 isolates are shown.

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vaccination with anti-pneumococcal vaccines was low to negligible and prior work indicated that these changes were due to a combination of secular trends and herd effect from children vaccination, which although occurring through the private market reached a coverage of 75% of children $\leq$2 yrs in 2008 [1,12,21]. Due to these changes one could expect that CC156 would

Table 3. Sequence types that were associated with pilus island 1 (PI-1) and pilus island 2 (PI-2).

Type of Pilus	ST	Yes	No	OR[a] (95% CI)	P-value[b]
Pilus 1	156	26	9	24.68 (10.79–61.89)	<0.001
	143	12	0	Inf (20.32-Inf)	<0.001
	179	10	1	72.89 (10.18–3133.46)	<0.001
	416	8	0	Inf (12.04-Inf)	<0.001
	162	6	0	Inf (8.16-Inf)	<0.001
	205	6	0	Inf (8.16-Inf)	<0.001
	2944	5	0	Inf (6.30-Inf)	<0.001
	1221	5	0	Inf (6.30-Inf)	<0.001
	4575	3	0	Inf (2.8-Inf)	0.002
	838	3	0	Inf (2.8-Inf)	0.002
	191	0	86	0 (0–0.27)	<0.001
	306	0	61	0 (0–0.39)	<0.001
	180	0	60	0 (0–0.40)	<0.001
Pilus 2	191	86	0	Inf (181.20-Inf)	<0.001
	306	61	0	Inf (99.04-Inf)	<0.001
	350	5	0	Inf (3.81-Inf)	<0.001
	180	0	60	0 (0–0.24)	<0.001
	53	0	39	0 (0–0.39)	<0.001
	156	0	35	0 (0–0.44)	<0.001

[a] OR—Odds ratio.

[b] Only significant values after FDR correction are shown.

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Table 4. Sequence types that were positively associated with penicillin non-susceptibility, erythromycin resistance, erythromycin and penicillin non-susceptibility simultaneously and multi drug resistance.

Antimicrobial resistance[a]	ST	Yes	No	OR[b] (95% CI)	P-value[c]	Penicillin MIC range (μg/ml)
PNSP	156	34	1	153.83 (25.29–6036.47)	<0.001	0.5–3
	276	31	0	Inf (34.58-Inf)	<0.001	0.19–3
	143	12	0	Inf (10.84-Inf)	<0.001	0.75–3
	338	10	0	Inf (8.65-Inf)	<0.001	0.064–0.19
	63	8	0	Inf (6.52-Inf)	<0.001	0.094–1
	386	7	0	Inf (5.48-Inf)	<0.001	0.064–0.19
	179	7	4	6.69 (1.68–31.50)	<0.001	0.047–2
	230	6	0	Inf (4.45-Inf)	<0.001	0.38–0.75
	2944	5	0	Inf (3.45-Inf)	<0.001	2–8
ERP	276	30	1	150.50 (24.56–5946.55)	<0.001	0.19–3
	179	11	0	Inf (10.96-Inf)	<0.001	0.047–2
	143	10	2	21.90 (4.60–206.95)	<0.001	0.75–3
	717	9	0	Inf (8.52-Inf)	<0.001	0.008–0.032
	9	8	0	Inf (7.32-Inf)	<0.001	0.016–0.064
	63	8	0	Inf (7.32-Inf)	<0.001	0.094–1
	386	7	0	Inf (6.15-Inf)	<0.001	0.064–0.19
	350	5	0	Inf (3.87-Inf)	<0.001	0.004–0.023
	230	5	1	21.27 (2.36–1006.44)	0.001	0.38–0.75
EPNSP	276	30	1	274.20 (44.39–10466.80)	<0.001	0.19–3
	143	10	2	36.81 (7.69–350.14)	<0.001	0.75–3
	63	8	0	Inf (12.17-Inf)	<0.001	0.064–0.19
	386	7	0	Inf (10.19-Inf)	<0.001	0.064–0.19
	179	7	4	12.52 (3.12–59.31)	<0.001	0.047–2
	230	5	1	35.15 (3.88–1669.53)	<0.001	0.38–0.75
	4575	3	0	Inf (2.83-Inf)	0.002	2–3

[a] PNSP—Penicillin non-susceptible pneumococci, ERP—Erythromycin resistant pneumococci, EPNSP—Erythromycin and penicillin non-susceptible pneumococci.

[b] OR—odds ratio. Inf—infinite.

[c] Only significant values after FDR correction are shown.

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also decrease (this CC accounted for 21.7% of all IPD in 1999–2003 [11]) and potentially lose its dominance. During the study period CC156 accounted for an approximately constant proportion of the characterized isolates in each year (varying slightly between 5.5% and 7.0%). The observed persistence of this CC may be explained by three different factors: 1) while it is true that PCV7 serotypes have declined in importance, it is also true that they still account for approximately one fifth of adult IPD and 57% of the isolates expressing PCV7 serotypes in 2008–2011 belonged to this CC; 2) this CC is strongly associated with antimicrobial resistance, with n = 70/101 isolates being resistant to at least two different classes of antibiotics; and 3) the genomic diversity of CC156 is high, with one study reporting the presence of 10 unrelated genetic subgroups [22], suggesting that this CC may be particularly suited to adapt to different selective pressures. Regarding the last point, in our study we found representatives of three different clones recognized by the PMEN included in CC156: Spain^{9V}-156, Colombia^{23F}-338 and Greece^{6B}-273 [20].

Overall, the clones recognized by the PMEN were strongly represented in our collection with up to 67.3% of the isolates being at most DLVs of one of the 29 different PMEN clones identified. Among the 22 major CCs occurring in the study period (Table 1), only six did not include a PMEN clone: CC433, CC378, CC460, CC260, CC30 and CC404. The most frequent of these, CC433 (mainly ST433, Table 1), was the eighth most frequent CC, included mostly isolates susceptible to antimicrobials, and is now an important cause of IPD worldwide [23–27].

The eight more frequent CCs (Table 1) were mainly composed of isolates expressing one of the top 10 serotypes causing adult IPD in 2008–2011, excluding serotype 4 that presented a high genetic diversity and no dominant CC (Fig 1). In fact, the clonal composition of the 10 most frequent serotypes causing adult IPD in Portugal in 2008–2011 (Figs 1 and 2) presented both similarities and differences with other geographic regions in similar periods, with most matching results coming from countries in Europe and the Americas, especially for serotypes 3 (Netherlands^{3}-180), 7F (Netherlands^{7F}-191), 22F (ST433) and 9N (ST66) [23–26,28–30]. Most of these lineages, with the exception of ST66, were also dominant among isolates expressing the same serotypes and causing IPD in children in Japan [27]. Among the isolates expressing serotypes 19F and 23F, the lineages that dominated in the present study where either absent or represented a minority of the isolates of the same serotype in the recent studies from the United States and Japan [26,27], indicating the persistence of different lineages expressing PCV7 serotypes in different countries. Serotype 19A, which increased as a cause of IPD after PCV7 implementation in several countries, was associated in Portugal with the expansion of the PMEN clone Denmark^{14}-230 while in the USA and Asia it was associated with the emergence of the PMEN clone Taiwan^{19F}-236, as previously described [3]. Serotype 1 was mostly represented by the Sweden^{1}-306 European clone [31]. However, we detected for the first time in Portugal two serotype 1 isolates belonging to the hypervirulent PMEN clone Sweden^{1}-217 (STs 217 and 3081), which has been responsible for epidemics with high mortality in Africa [31,32]. The detection of these genotypes in Portugal is not surprising, since they were found in neighboring Spain [28] and Portugal has a significant community of citizens of African descent. Still, the two isolates detected were collected in 2011, the last year of the study period, so it will be important to monitor the potential emergence of this genotype as a cause of adult IPD in Portugal. Serotypes 14 and 8 were found mainly among representatives of Spain^{9V}-156 and Netherlands^{8}-53, respectively, similarly to Spain [28]. Serotype 11A was found mainly among representatives of ST408 in our study, while the most common lineage in both Spain and the USA was its SLV, ST62 [26,28]. For serotype 4, in spite of the higher diversity some similarity was also found with Spain, with Sweden^{4}-205 and ST246 being common to the two collections of isolates [28].

When comparing our results with those from a recent carriage study in adults in Portugal [33] in addition to the difference in serotype distribution due to the recognized differences in invasiveness of the various serotypes [2], there was also a marked difference between the clonal compositions of serotype 19A, since the majority of isolates expressing this serotype among asymptomatic carriers represented ST1201 (CC15), while in our study the most frequent was ST276, indicating possible differences in virulence between these two serotype 19A lineages.

After the introduction of PCV7, several studies documented a general decrease in IPD incidence. However, the benefits of vaccination were also partly overcome by increases in incidence of non-vaccine serotypes [5,7,34,35]. This could occur through the persistence of a successful lineage now expressing a different serotype not covered by the conjugate vaccines, a phenomenon described as capsular switching. Among our collection a notable case of possible capsular switching was the detection of five isolates related to the PMEN clone Denmark^{14}-230 (ST230, n = 4 and ST4253, n = 1) expressing the non-PCV13 serotype 24F (Table 2). This combination

has already been reported in Portugal in colonized children [36], in Italy [37], Spain and other European countries (http://pubmlst.org/). In Portugal, in the pre-PCV7 period, serotype 24F was predominantly CC81 and mostly susceptible to antimicrobials. In 2008–2011, among the nine isolates genotyped, four represented CC81 and were mainly antimicrobial susceptible as before, while five represented CC230 and were EPNSP. The detection of this genotype expressing serotype 24F in Portugal is of concern since ST276, an SLV of ST230, was behind the expansion of serotype 19A as a cause of IPD in Portugal in the post-PCV7 era [3]. Among other possible capsular switches detected in our collection (Table 2), most reflected the occasional detection of a single isolate of a different serotype, suggesting that even if these result from capsular switching they did not persist in the population at a significant frequency. Taken together this data indicates that capsular switching in our collection was infrequent and cannot be attributed to vaccine pressure, in agreement with other studies [38,39]. However, even though these events were rare they can be important since the uncommon combinations may proliferate in the future if the conditions become favorable maintaining successful clones in circulation.

Clonal expansion of previously less frequent lineages was a major contributor to the expansion of non-PCV7 serotypes, since the 22 most frequent CCs occurring in 2008–2011 (Table 1) were already in circulation in 1999–2003 [11]. When comparing these two periods the most relevant changes were the expansion of CC191 (serotype 7F) and CC439 (serotypes 23B and 23A) and the decline of CC260 and CC458 (both associated with serotype 3), CC1381 (serotype 18C) and that of CC156 discussed above. The variations in frequency of CC191, CC439 and CC1381 followed the changes occurring in the respective serotypes. Regarding the clonal composition of serotype 3, we found that the decrease in CC260 and CC458 was accompanied by an expansion of CC180 among serotype 3 isolates, explaining the relative stability of this serotype among IPD in adults [12], with CC180 accounting for 40% of serotype 3 IPD in 1999–2003 but for 64% in 2008–2011. Given that isolates belonging to CC180, CC260 or CC458 were mostly susceptible to all tested antimicrobials and that only one isolate from CC180 and another from CC458 carried a PI, this different behavior in time cannot be attributed to differences in these characteristics.

The presence and type of the PIs was more strongly associated with genotype than with serotype, as previously reported [4]. The genotypes that carried PIs in our study (Table 3) were essentially the same reported recently in USA [26], although the proportions of these genotypes differed considerably between the two studies. The proportion of PI-1 carrying isolates increased in the post-PCV7 period in the USA associated with the emergence of the non-PCV7 serotypes 19A and 35B [40]. Although serotype 19A also increased in Portugal, the genotype behind this increase does not carry a PI (ST276) and an actual decrease of PI-1 positive isolates occurred when compared to the pre-PCV7 period, when 24% of the adult isolates presented PI-1 [4]. The proportion of isolates presenting only PI-2 declined during the study period, from 25% in 2008 to 15% in 2011. This was expected since serotype 1 isolates are significantly associated with PI-2 and these decreased as a cause of adult IPD during the study period [12]. Since PCV13 also includes serotype 7F, which in Portugal was strongly associated with PI-2, continued use of PCV13 may further reduce the proportion of isolates carrying PI-2. In 2011, the proportion of isolates carrying any of the PIs was down to 26.6% of the isolates. As suggested for isolates causing IPD in children [16], continued PCV13 use has the potential to virtually eliminate PI carrying isolates.

Antimicrobial resistance is not a crucial pre-requisite for the success of serotypes in IPD, as demonstrated by serotypes 1, 3 and 7F that were frequent in the post-PCV7 period and are mostly susceptible to antimicrobials. Still, the presence of resistant clones may help the persistence of serotypes targeted by vaccines, as was possibly the case with serotypes 14 and 19A.

The highest proportions of penicillin and erythromycin resistance among adult IPD since the beginning of epidemiological surveillance were registered in 2010, although these declined again in 2011 [12]. Between 2008 and 2009, when only the increase in PNSP was significant, this was due to an increase in PNSP expressing serotypes 14 and 19A. In contrast, between 2009 and 2010, the increase in both PNSP and ERP was due to an increase in genetically unrelated resistant isolates expressing different serotypes. Since the number of isolates collected yearly between 2008 and 2011 did not suffer significant fluctuations, two possibilities could explain the initial increase in PNSP isolates expressing serotypes 14 and 19A: 1) an increase in the overall proportions of serotypes 14 and 19A, including PNSP STs or 2) an increase in the proportion of PNSP STs within each of these serotypes, with a concomitant decrease of susceptible STs. Regarding serotype 14 isolates, which increased slightly during the study period, these were by 2008 almost equally distributed into only two CCs: CC15, which includes ST409 and that is almost entirely penicillin susceptible, and CC156, in which all serotype 14 isolates were PNSP. From 2009 onwards, CC156 became the dominant lineage, accounting for over 90% of the isolates expressing serotype 14, a change that was not only due to a decline in frequency of CC15 but also to a slight overall increase in frequency of CC156 among all adult IPD isolates. Among serotype 19A isolates, the increase in proportion of PNSP between 2008 and 2009 was due to the disappearance of ST193, which was fully susceptible to penicillin, and to an increase of ST276, which represented solely PNSP isolates (Table 4). Although PNSP and ERP returned in 2011 to values similar to those found prior to 2010, this was due to a decrease in frequency of resistant isolates representing multiple STs and expressing different serotypes, while the emerging clones (CC156 among serotype 14 and ST276 among serotype 19A) persisted as important causes of adult IPD. Continued surveillance of resistant isolates should focus particularly on the evolution of serotype 24F since $\geq$50% of the isolates expressing this serotype in our study were associated with the PMEN clone Denmark^{14}-230 (S2 Table) which was a major clone in the expansion of serotype 19A in the post-PCV7 period in Portugal.

The significant differences in genetic variation, as documented here by MLST, within the various serotypes remain unexplained and should be the object of future study. We have shown that the changes in serotypes occurring during the study period have been driven mostly by the expansion of previously circulating clones or to declines in the majority of the lineages expressing a given serotype. However, in some serotypes, such as 14 and 19A, changes in serotype frequency were driven mostly by changes in particular lineages. In the case of serotype 3, although its proportion remained constant with time, there were significant changes in the dominant lineages. These observations raise the possibility that lineage-specific properties may condition the dynamics of particular serotypes. Serotype switching played a minor role in this population but may be an important source of new variants that may increase in the post PCVs period. Taken together, these observations reinforce the importance of determining the clonal lineages of pneumococci to better understand the changes in the bacterial population occurring following the use of PCVs.

Supporting Information

S1 Table. Age distribution and serotypes of the STs found in CCs with less than 10 isolates.
(PDF)

S2 Table. Distribution of STs according to serotype of the isolates (n$\leq$11) causing adult IPD in 2008–2011 and expressing serotypes not included in any of the conjugate vaccines.
(PDF)

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Author Contributions

Conceived and designed the experiments: JMC MR. Performed the experiments: ANH CSC JDM JPL. Analyzed the data: ANH JMC MR. Contributed reagents/materials/analysis tools: PGSSI. Wrote the paper: ANH CSC JMC MR.

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Non-Invasive Pneumococcal Pneumonia in Portugal—Serotype Distribution and Antimicrobial Resistance

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Abstract

There is limited information on the serotypes causing non-invasive pneumococcal pneumonia (NIPP). Our aim was to characterize pneumococci causing NIPP in adults to determine recent changes in serotype prevalence, the potential coverage of pneumococcal vaccines and changes in antimicrobial resistance. Serotypes and antimicrobial susceptibility profiles of a sample of 1300 isolates recovered from adult patients (≥18 yrs) between 1999 and 2011 (13 years) were determined. Serotype 3 was the most frequent cause of NIPP accounting for 18% of the isolates. The other most common serotypes were 11A (7%), 19F (7%), 19A (5%), 14 (4%), 22F (4%), 23F (4%) and 9N (4%). Between 1999 and 2011, there were significant changes in the proportion of isolates expressing vaccine serotypes, with a steady decline of the serotypes included in the 7-valent conjugate vaccine from 31% (1999–2003) to 11% (2011) ($P<0.001$). Taking together the most recent study years (2009–2011), the potential coverage of the 13-valent conjugate vaccine was 44% and of the 23-valent polysaccharide vaccine was 66%. While erythromycin resistance increased from 8% in 1999–2003 to 18% in 2011 ($P<0.001$), no significant trend was identified for penicillin non-susceptibility, which had an average value of 18.5%. The serotype distribution found in this study for NIPP was very different from the one previously described for IPD, with only two serotypes in common to the ones responsible for half of each presentation in 2009–2011 – serotypes 3 and 19A. In spite of these differences, the overall prevalence of resistant isolates was similar in NIPP and in IPD.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Introduction

Pneumonia is a common infection that causes high rates of morbidity and mortality worldwide. *Streptococcus pneumoniae* (pneumococcus) is thought to be the major cause of pneumonia, responsible for up to half of all cases [1]. Only a small fraction of pneumococcal pneumonias are bacteremic, with non-invasive pneumococcal pneumonia (NIPP) estimated to be three to ten-times more frequent than invasive pneumococcal pneumonia [2,3]. While bacteremic pneumonia is a more severe form of pneumonia, it is less clear if bacteremia can be considered an independent predictor of mortality [4,5]. In adults, bacteremic pneumonia accounts for most of the cases of invasive pneumococcal disease (IPD). While the serotype distribution of IPD and NIPP have been sometimes assumed to be the same [6], it is becoming increasingly clear that this is not so [5,7]. This observation is in agreement with the recognition that some serotypes, and even different genetic lineages expressing the same serotype, may have different invasive disease potentials [8], leading to the expectation that less invasive serotypes would be more abundantly represented in NIPP than in IPD.

In developed countries, pneumonia is believed to be a major cause of morbidity among older adults, and, together with influenza, is the leading cause of death from infectious disease in the US considering the entire population [9]. Until recently, the only available vaccine for adults was the 23-valent polysaccharide vaccine (PPV23) that, while potentially effective in preventing IPD, may be less efficacious against NIPP [10,11]. Possibly due to the ongoing debate on the usefulness of PPV23 vaccination, in the majority of the European countries, including Portugal, there has been a low uptake of this vaccine [12,13]. On the other hand, the 7-valent conjugate vaccine (PCV7) was introduced in many European countries for vaccinating children, rapidly reaching high coverages in the targeted age groups. PCV7 was available in Portugal for vaccination of children between 2001 and 2009 and, although not part of the national immunization program, its uptake was estimated to have grown continuously, albeit slower that in countries where it was part of the national immunization

program [14]. Changes in the serotype distribution of isolates causing IPD, compatible with an effect of PCV7 occurred in both children and adults in Portugal and elsewhere [13–17], the latter potentially resulting from a herd effect. The 10-valent (PCV10) and the 13-valent conjugate vaccines (PCV13) became available in Portugal in 2009 and in 2010, respectively. In September 2011, PCV13 received the European Medicines Agency approval for use in adults ≥50 yrs and in July 2013 was approved for adults ≥ 18 yrs, for the prevention of IPD. Currently PCV13 is approved for all ages from 6 weeks up and there is now initial evidence of its efficacy against pneumococcal pneumonia in adults caused by the serotypes included in the vaccine [18].

The aim of this study was to determine the serotype distribution and antimicrobial resistance of pneumococci causing NIPP in adults in Portugal during a 13-year period, when the three conjugate vaccines were available for children, and to compare these data to the information available for IPD published previously [19].

Materials and Methods

Ethics Statement

Case reporting and isolate collection were considered to be surveillance activities and were exempt from evaluation by the Review Board of the Faculdade de Medicina da Universidade de Lisboa. The data and isolates were de-identified so that these were irretrievably unlinked to an identifiable person.

Bacterial Isolates

Isolates were provided by a laboratory-based surveillance system that includes 30 microbiology laboratories throughout Portugal. These were asked to identify and send to our laboratory all pneumococci causing infections. Although the laboratories were contacted periodically to submit the isolates to the central laboratory, no audit was performed to ensure compliance, which may be variable in this type of study. After arrival, all isolates were confirmed as *S. pneumoniae* by colony morphology and hemolysis on blood agar plates, optochin susceptibility and bile solubility. The isolates included in this study were recovered from adult patients (≥18 yrs) with a clinical diagnosis of pneumonia between 1999 and 2011. A total of 1300 isolates, 100 isolates randomly chosen from among the isolates received in each of the 13 years of the study were included. The total number of isolates submitted to the central laboratory in each year was 161 in 1999, 184 in 2000, 319 in 2001, 282 in 2002, 265 in 2003, 341 in 2004, 338 in 2005, 392 in 2006, 525 in 2007, 601 in 2008, 473 in 2009, 519 in 2010 and 445 in 2011. We believe this reflects increasing compliance with the surveillance activities with time. Only isolates recovered from sputum, bronchial secretions or bronchoalveolar lavage were considered. Isolates were not included when pneumococci were simultaneously isolated from blood or another usually sterile product, and when other potential bacterial pathogens besides pneumococci were detected in the sample (such as *Haemophilus influenzae* that was frequently detected). Only one isolate from each patient in each year was considered. Among the 1300 isolates selected, 103 (7.9%) were isolates from bronchoalveolar lavage fluid.

Serotyping and Antimicrobial Susceptibility Testing

Serotyping was performed by the standard capsular reaction test using the chessboard system and specific sera (Statens Serum Institut, Copenhagen, Denmark). Serotypes were grouped into conjugate vaccine serotypes, i.e., those included in PCV13 (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 19A, 23F) that comprise all the serotypes found in the lower valency vaccines (PCV7: 4, 6B, 9V, 14, 18C, 19F, 23F; and PCV10: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F), those included in PPV23 (all serotypes included in PCV13 except 6A and serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F), and non-vaccine serotypes (NVT). The isolates that were not typable with any of the complete set of sera were considered non-typable (NT).

Minimum inhibitory concentrations (MICs) for penicillin and cefotaxime were determined using Etest strips (Biomérieux, Marcy-L'Etoile, France). The results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) recommended breakpoints prior to 2008 [20], as these allows the comparison with previously published data. According to these criteria, intermediate level penicillin resistance is defined as MIC 0.12–1.0 μg/ml and high level resistance as MIC≥2.0 μg/ml. Isolates that fell into either one of these classes were designated penicillin non-susceptible. Susceptibility to cefotaxime was defined as MIC≤ 1.0 μg/ml. The Kirby-Bauer disk diffusion assay was used to determine susceptibility to levofloxacin, erythromycin, clindamycin, chloramphenicol, trimethoprim/sulphamethoxazole, tetracycline, vancomycin and linezolid, according to the CLSI recommendations and interpretative criteria [21]. Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin, as previously described [22]. The MLS_B phenotype (resistance to macrolides, lincosamides and streptogramin B) was defined as the simultaneous resistance to erythromycin and clindamycin, while the M phenotype (resistance to macrolides) was defined as non-susceptibility only to erythromycin.

Statistical Analysis

Sample diversity was measured using Simpson's index of diversity (SID) and the respective 95% confidence intervals ($CI_{95\%}$) [23]. To compare two sets of partitions the Adjusted Wallace (AW) coefficients were calculated [24] using the online tool available at www.comparingpartitions.info. Differences were evaluated by the Fisher exact test with the false discovery rate (FDR) correction for multiple testing [25] or the Chi-squared test, and the Cochran-Armitage test was used for trends. A $P<0.05$ was considered significant for all tests.

Results

Serotype Distribution

Serotype diversity was high [SID: 0.941, $CI_{95\%}$: 0.935–0.948], with 57 different serotypes detected among the 1300 isolates. The most frequent serotypes, which accounted for more than half of the isolates, were serotypes: 3 (17.8%), 11A (6.7%), 19F (6.7%), 19A (5.2%), 14 (4.1%), 22F (4.1%), 23F (3.8%) and 9N (3.5%). Serotype distribution in each of the studied years is represented in Figure 1. We chose to represent an average of the yearly values between 1999 and 2003, because it was shown previously that this period corresponded to the years before an effect of children vaccination with PCV7 was noted in the distribution of adult IPD serotypes [14]. The yearly distribution on the 10 overall most frequent serotypes between 1999 and 2003 is represented in Figure S1. In spite of yearly variations, serotype diversity was high in all studied years with the lowest SID detected in 2008 [SID: 0.901, $CI_{95\%}$: 0.857–0.945] and the highest value found in both 2004 and 2009 [SID: 0.957, $CI_{95\%}$: 0.840–0.973].

The change in distribution of vaccine types along the study years is shown in Figure 2 and Figure S2. The serotypes included in PCV7 declined gradually from 31% in 1999–2003 to 11% in 2011 (Cochran-Armitage test of trend $P<0.001$). Among the

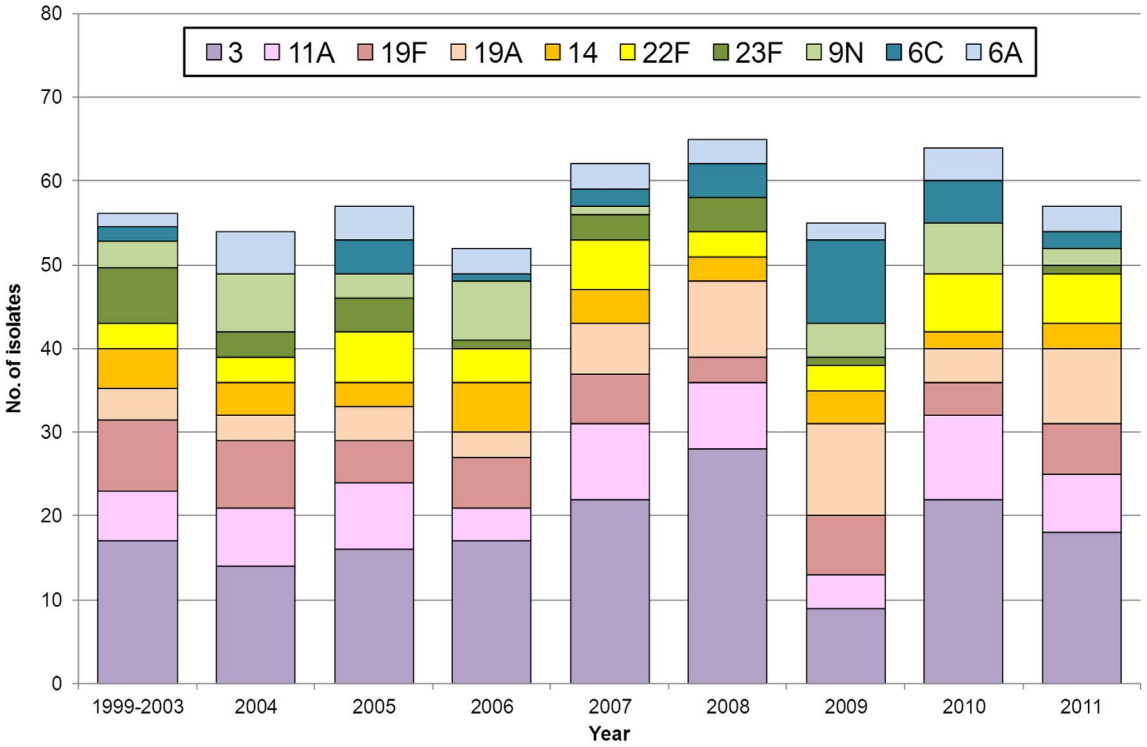

Figure 1. Serotype distribution of the isolates causing non-invasive pneumococcal pneumonia in adults in Portugal (1999–2011). Only the overall 10 most frequent serotypes are shown. The other serotypes found between 1999 and 2011 were serotypes 6B, 7F and 15A (n = 32, each), 23B (n = 30), 15B (n = 28), 10A (n = 27), 9V and non-typable (n = 26, each), 23A (n = 24), 1 (n = 22), 8 (n = 20), 16F (n = 19), 29 (n = 18), 4 (n = 17), 31 (n = 16), 34 (n = 15), 18C (n = 14), 17F and 33A (n = 13, each), 21 and 35F (n = 11, each), 15C and 35C (n = 10, each), 20 (n = 9), 12B and 17A (n = 8, each), 13, 25F and 25A/38 (n = 7, each), 7C and 28A (n = 5, each), 5 (n = 4), 11F, 18A and 19C (n = 3, each), 12A, 12F, 35A, 35B, 38 (n = 2, each) and 9L, 10F, 15F, 16A, 19B, 33F, 39 and 42 (n = 1, each). The value shown for 1999–2003 refers to the yearly average of the 500 isolates studied that were isolated in these 5 years. This period was analyzed together since previously published IPD data indicated that these corresponded to a pre-PCV7 serotype distribution [14].
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PCV7 serotypes, those that contributed mostly to this decline were serotypes 6B (from 4.6% to 0%, Cochran-Armitage test of trend $P<0.001$), 9V (from 3.2% to 0%, Cochran-Armitage test of trend $P<0.001$) and 23F (from 6.6% to 1.0%, Cochran-Armitage test of trend $P<0.001$). Despite fluctuations throughout the study period in the number of isolates representing PCV13 and PPV23 serotypes, no consistent trend was noted. However, a decline of the isolates expressing serotypes included in these vaccines, and a consequent increase in the prevalence of NVTs, can be distinguished between 2008 and 2009. This can be attributed to a fall in serotype 3, from 28% to 9%, between these two years ($P< 0.001$, Figure 1). Although in subsequent years the proportion of isolates expressing serotype 3 returned to values similar to those found previously (Figure 1), this did was not reflected in an increase in the proportion of isolates expressing PCV13 serotypes, which remained close to 43% (Figure 2). In contrast, the decline in the proportion of isolates expressing PPV23 serotypes noted in 2009 was not sustained, with the increases in the following years bringing this value back into the range found in the previous decade (Figure 2).

The distribution of the 10 most frequent serotypes found between 1999 and 2011, stratified by age group, is shown in table S1. The serotype distribution is similar for each of the age groups considered ($P = 0.398$) and no significant associations, after correction for FDR, could be found between specific serotypes and age groups. When considering only the three last years of the study, corresponding to the years immediately prior to PCV13 receiving approval for use in adults (2009–2011), the overall proportion of isolates expressing serotypes included in the various vaccines were 10.3% for PCV7, 43.7% for PCV13 and 66.0% for PPV23. There was also no correlation between the proportion of isolates causing NIPP included in the vaccines and the different age groups (table S2).

Antimicrobial susceptibility

The proportion of isolates resistant to the tested antimicrobials between 1999 and 2011 is summarized in table 1. Penicillin non-susceptible pneumococci (PNSP) accounted for 18.5% of the isolates (n = 241). Among these, 211 isolates (16.2%) expressed low level resistance (MIC = 0.12–1 μg/mL) and 30 isolates (2.3%) high level resistance (MIC≥2 μg/mL). Considering the current CLSI guidelines for parenteral penicillin in non-meningitis cases, where high level resistance is defined as MIC≥8 μg/mL and intermediate resistance as MIC≥2 μg/mL [21], only 16 strains (1.2%) would have been considered non-susceptible to penicillin, with only one of these expressing high level resistance.

Erythromycin resistant pneumococci (ERP) accounted for 16.3% of the isolates (n = 212), with 84.0% (n = 178) of these expressing the MLS_B phenotype and 16.0% (n = 34) the M phenotype. A total of 9.8% (n = 127) of the isolates were simultaneously non-susceptible to penicillin and resistant to erythromycin (EPNSP).

Resistance to levofloxacin was low overall (1.3%, n = 17), but higher in the older age groups than in the youngest group

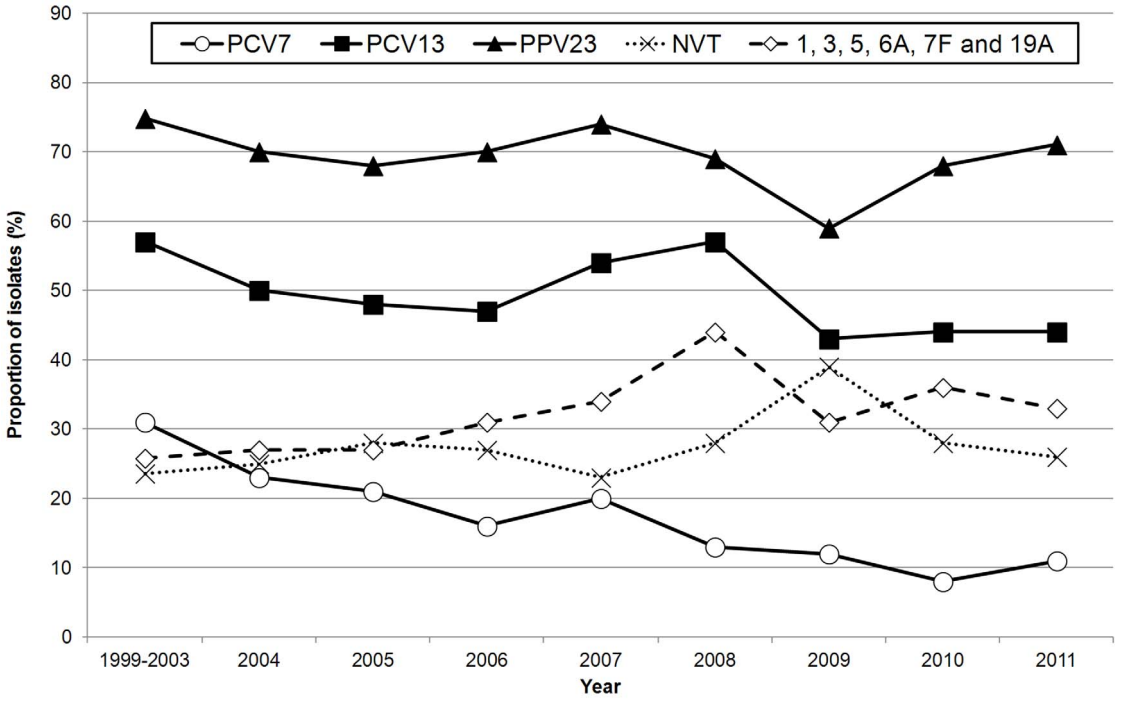

Figure 2. Proportion of isolates expressing serotypes included in pneumococcal vaccines causing non-invasive pneumococcal pneumonia in adults in Portugal (1999–2011). The value shown for 1999–2003 refers to the yearly average of the 500 isolates studied that were isolated in these 5 years. This period was analyzed together since previously published IPD data indicated that these corresponded to a pre-PCV7 serotype distribution [14].
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(Table 1, 18–49 yrs versus 50–64 yrs, P = 0.014 and 18–49 yrs versus ≥65 yrs, P = 0.012). No other significant associations with age were found for the other antimicrobials tested. All isolates were susceptible to vancomycin and linezolid.

The proportion of erythromycin and clindamycin resistant isolates increased between 1999–2003 and 2011. Erythromycin resistance increased from 8.0% to 18.0% (Cochran-Armitage test of trend P<0.001) and clindamycin resistance increased from

Table 1. Antimicrobial resistance of the isolates responsible for non-invasive pneumococcal pneumonia in adults in Portugal, stratified by age groups (1999–2011).

	No. resistant isolates (%)[a]			
	[18–49] yrs	[50–64] yrs	≥65 yrs	Total
	(n = 481)	(n = 293)	(n = 526)	(n = 1300)
PEN[b]	95 (19.8)	57 (19.5)	89 (16.9)	241 (18.5)
MIC90	0.25	0.38	0.25	-
MIC50	0.023	0.023	0.023	-
CTX	4 (0.8)	3 (1.0)	2 (0.4)	9 (0.7)
MIC90	0.19	0.125	0.094	-
MIC50	0.012	0.012	0.012	-
LEV	1 (0.2)	6 (2.0)	10 (1.9)	17 (1.3)
ERY	76 (15.8)	54 (18.4)	82 (15.6)	212 (16.3)
CLI	66 (13.7)	44 (15.0)	68 (12.9)	178 (13.7)
CHL	17 (3.5)	12 (4.1)	22 (4.2)	51 (3.9)
SXT	89 (18.9)	46 (15.7)	84 (16.0)	219 (16.8)
TET	61 (12.7)	42 (14.3)	63 (12.0)	166 (12.8)

[a]PEN – penicillin; CTX – cefotaxime; LEV – levofloxacin; ERY – erythromycin; CLI – clindamycin; CHL – chloramphenicol; SXT – trimethoprim/sulphamethoxazole; TET – tetracycline. All isolates were susceptible to vancomycin and linezolid.
[b]Non-susceptibitily to penicillin was determined using the CLSI breakpoints prior to 2008 [20].
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7.0% to 15.0% (Cochran-Armitage test of trend P = 0.004). No other significant changes were noted for the other antimicrobials tested.

There was an association between serotype and antimicrobial resistance. The AW for serotype and PNSP was 0.588 ($CI_{95\%}$: 0.541–0.634) and the AW for serotype and ERP was 0.489 ($CI_{95\%}$: 0.419–0.558). Table 2 shows the serotypes that presented at least 10 PNSP and ERP isolates, respectively. Among the major serotypes expressed by PNSP, only serotype 19F was not significantly associated with PNSP. Among the major serotypes expressed by ERP, only serotypes 23F and 6C were not significantly associated with ERP. The PCV7, PCV13 and PPV23 serotypes accounted for 56.0%, 70.5% and 69.7% of PNSP, respectively, and 42.9%, 60.8% and 66.5% of ERP, respectively.

Discussion

Serotype 3 was the most important serotype in NIPP in adults in Portugal. This serotype was the most frequently detected in all studied years, with the exception of 2009, when it ranked third (Figure 1). A predominance of serotype 3 was also found in two studies that focused on the serotype distribution of pneumococcal pneumonia isolates [5,26], and this serotype was among the most frequent in two recent studies using urinary antigen detection assays to diagnose pneumococcal pneumonia [7,27], although these last studies included both NIPP and bacteremic pneumonia. In Portugal, serotype 3 is not only a leading cause of NIPP but also of IPD, as can be seen in Figure S3, showing the distribution of the most frequently detected serotypes immediately prior to the licensure of PCV13 for adult immunization (2009–2011).

Although both IPD and NIPP were characterized by a high serotype diversity (for NIPP SID = 0.943, $CI_{95\%}$: 0.932–0.955; and for IPD SID = 0.942, $CI_{95\%}$: 0.937–0.946; considering 2009–2011), the actual serotype distribution is quite different (Figures 3, Figure S3 and Table S3). Among the most frequent serotypes, accounting for half of the characterized isolates in 2009–2011, only two serotypes were common to both NIPP and IPD, which were serotypes 3 and 19A (Figure S3). When comparing the serotype distribution, serotypes 6A, 11A, 15C, 19F and 23B were significantly more abundant among NIPP isolates, whereas serotypes 1, 4,7F and 14 were significantly more abundant among IPD isolates (Figure 3 and Table S3). These four serotypes, together with serotype 3, were already shown to have an enhanced invasive disease potential in a study evaluating the serotypes and clones circulating in Portugal [8]. On the other hand, serotypes 6A, 11A and 19F were associated with carriage [8], suggesting their lower invasive disease potential, consistent with the association with NIPP determined here (Figure 3 and Table S3).

A recent study described the serotype distribution among isolates recovered in 2011 causing bacteremic and non-bacteremic pneumonia in adults in Denmark [5]. When considering only the isolates recovered in 2009–2011 in Portugal, the serotype distribution is, perhaps surprisingly, remarkably similar in both studies. Among the differences are the more significant fractions of non-typable isolates among NIPP and of serotype 1 isolates in bacteremic pneumonia in Denmark relative to Portugal. Serotype 1 was always an important serotype among isolates causing IPD in both children and adults in Portugal, but its significance has

Table 2. Serotype distribution of PNSP and ERP causing non-invasive pneumococcal pneumonia in adults in Portugal (1999–2011).

	Serotype[a]	No. of resistant isolates (%)	OR ($CI_{95\%}$)	P-value[b]
PEN	23F	39 (16.2)	12.6 (6.2–27.7)	**<0.001**
	14	37 (15.4)	8.1 (4.3–15.9)	**<0.001**
	19A	28 (11.6)	2.3 (1.3–3.9)	**0.002**
	15A	27 (11.2)	18.2 (6.8–61.3)	**<0.001**
	19F	27 (11.2)	1.4 (0.8–2.3)	0.193
	9V	23 (9.5)	25.5 (7.6–133.7)	**<0.001**
	6C	18 (7.5)	3.0 (1.5–6.2)	**0.001**
	Others[c]	42 (17.4)	-	-
ERY	19F	37 (17.5)	3.4 (2.1–5.4)	**<0.001**
	19A	31 (14.6)	3.7 (2.2–6.4)	**<0.001**
	15A	28 (13.2)	31.9 (11.0–126.2)	**<0.001**
	14	21 (9.9)	2.8 (1.5–5.1)	**<0.001**
	6B	15 (7.1)	3.7 (1.7–8.0)	**<0.001**
	23F	14 (6.6)	1.6 (0.8–3.1)	0.151
	33A	10 (4.7)	13.8 (3.5–79.0)	**<0.001**
	6C	10 (4.7)	1.5 (0.6–3.3)	0.296
	NT[d]	10 (4.7)	2.6 (1.0–6.1)	**0.025**
	Others[e]	36 (17.0)	-	-

[a]Only the serotypes that presented at least 10 non-susceptible isolates are shown.
[b]Significant P-values after FDR correction are highlighted in bold.
[c]Other serotypes found among PNSP: 6B (n = 8), non-typable (n = 7), 6A and 29 (n = 5, each), 23B and 24F (n = 3, each), 7C (n = 2), 1, 3, 4, 11A, 15B, 15F, 22F, 23A, 35A (n = 1, each).
[d]NT – non typable.
[e]Other serotypes found among ERP: 9V and 11A (n = 4, each), 3, 15B, 22F, 23A, 24F (n = 3, each), 6A (n = 2), 1, 7F, 8, 9N, 15F, 16F, 17F, 23B, 29, 33F and 35F (n = 1, each).
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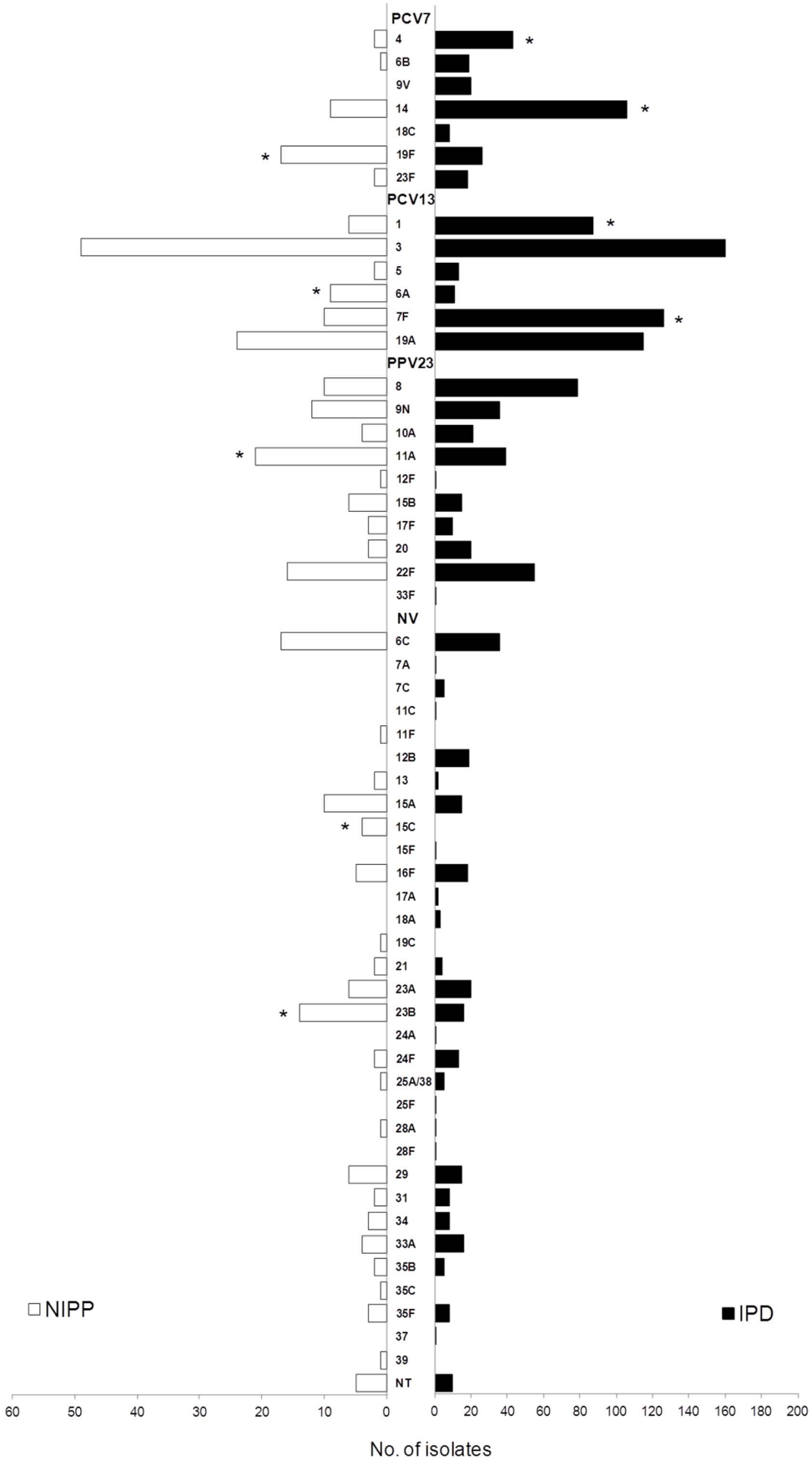
PCV7
4
6B
9V
14
18C
19F
23F
PCV13
1
3
5
6A
7F
19A
PPV23
8
9N
10A
11A
12F
15B
17F
20
22F
33F
NV
6C
7A
7C
11C
11F
12B
13
15A
15C
15F
16F
17A
18A
19C
21
23A
23B
24A
24F
25A/38
25F
28A
28F
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33A
35B
35C
35F
37
39
NT
NIPP
IPD
60 50 40 30 20 10 0
0 20 40 60 80 100 120 140 160 180 200
No. of isolates

Figure 3. Serotype distribution of the isolates causing non-invasive pneumococcal pneumonia and invasive pneumococcal disease in adults in Portugal (2009–2011). Data from IPD were published previously [19]. Serotypes associated with NIPP or IPD are marked by asterisks. The odds ratio was used to measure the association between serotype and disease presentation and only significant values ($P<0.05$) after FDR correction are indicated. The P values for the serotypes associated with NIPP were: $P = 0.001$ for 19F, $P = 0.007$ for 6A, $P = 0.004$ for 11A, $P = 0.001$ for 15C, $P<0.001$ for 23B. The P values for the serotypes associated with IPD were: $P = 0.007$ for 4, $P<0.001$ for 14, $P<0.001$ for 1 and $P<0.001$ for 7F.
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declined in recent years [13,15,19,28]. This change cannot be attributed entirely to the use of higher valency vaccines, such as PCV13, although vaccination was potentially a contributing factor [19,28]. Another important difference is the persistence of serotype 14 isolates among both NIPP and IPD in Portugal, in contrast to Denmark where this serotype was found at a lower frequency [5]. Serotype 14 was already included in PCV7, and continued vaccine use could be expected to significantly reduce its prevalence. A lower and more protracted vaccine uptake in Portugal compared to Denmark, together with a higher antibiotic consumption, could contribute to the differences observed between the two countries.

In previous studies, we showed that immunization of children with PCV7 resulted in the decline of PCV7 serotypes as causes of adult IPD [13,19]. In the present study, we show that a decline of PCV7 serotypes also occurred among isolates causing NIPP in adults. However, while in IPD this decline was abrupt, occurring between 2004 and 2005, in NIPP this decline was gradual and occurred over the entire post-PCV7 period (Figure 2 and Figure S2). The proportion of isolates expressing PCV7 serotypes in the most recent years of the study was different between NIPP and IPD (10.3% versus 19.0% in 2009–2011, $P<0.001$, Figure 3).

Taken together, the isolates expressing PCV13 serotypes also declined in IPD and in NIPP, a change that was observed from 2008 onwards for both disease presentations. However, again there were important differences. While in IPD this decline occurred from 2008 to 2011 and was mostly due to decreases in serotypes 1 and 5, in NIPP this decline occurred between 2008 and 2009 and was predominantly caused by a decrease in serotype 3. In neither case can we attribute these changes to the introduction of PCV13 in Portugal, since this vaccine only became available for children in the beginning of 2010 and received an indication for adults in the beginning of 2012. In NIPP, one possible explanation for the change observed could be the H1N1pdm09 pandemic that occurred between 2009 and 2010. Individuals infected by influenza are at high risk of developing secondary bacterial infections, especially with pneumococci [29]. Consistent with the hypothesis that influenza allowed the emergence of multiple serotypes as causes of NIPP, the decrease of serotype 3 from 2008 to 2009 was accompanied by an increase in serotype diversity.

Another remarkable difference between NIPP and IPD is the proportion of isolates that expresses serotypes included in the available vaccine formulations with an adult indication. When analyzing data from 2009 to 2011, we found that the number of IPD cases that could have been potentially prevented by PCV13 and PPV23 was 59%, and 80%, respectively [19], while the proportion of isolates expressing these serotypes was only 44% and 66%, respectively, among our collection of NIPP isolates. The higher proportion of vaccine types among IPD isolates was also documented in Denmark [5]. The efficacy of the conjugate vaccines is well established and adults could now benefit directly from PCV13 use. However, according to our sample more than half of NIPP cases could not have been prevented by vaccination with PCV13.

In the present study we could not find any significant associations between serotypes and age groups. This is in contrast to our previous studies with invasive isolates, where serotypes 3 and 19A were associated with older patients and serotypes 1 and 8 were associated with younger patients [19]. However, if we do not consider the correction for multiple testing, serotype 3 was more frequent in older adults than in the youngest (14% in 18–49 yrs versus 20% in $\geq$50 yrs, $P = 0.005$, Table S1). The lack of association for the other three serotypes with age is likely the result of their small numbers in our NIPP sample, particularly in what concerns serotypes 1 and 8.

A high proportion of the resistant isolates recovered between 1999 and 2011 are of serotypes included in PCV7 (Table 2), accounting for 56% of PNSP and 43% of ERP in the entire study period. Unlike what could have been expected, the introduction of PCV7 in Portugal did not reduce the proportion of resistant isolates, neither in NIPP nor in IPD [14,19]. Actually, for both presentations there was an increase in ERP between 1999 and 2011, and for IPD there was also an increase in PNSP. However, when we considered the most recent data (2009 to 2011) we found that only 22% of PNSP and 26% of ERP causing NIPP, represented serotypes included in PCV7. This means that resistant isolates expressing serotypes that are not included in PCV7 have emerged and expanded in the post-PCV7 period.

When considering the entire study period, antimicrobial resistance among NIPP isolates was similar to the values reported recently for IPD [19] (Table 1). Given the association between serotype and antimicrobial resistance, and the different serotype distributions between NIPP and IPD, how can we explain the similar overall resistance? For the most part, the explanation can be found in the more gradual decrease of resistant PCV7 serotypes, albeit to a lower level, in NIPP when compared to IPD. This was accompanied by the rise of a different set of serotypes including resistant isolates that are not included in PCV7, resulting in similar overall resistance levels. Resistance among NIPP isolates is partly due to the proliferation of resistant serotype 19A isolates, probably representing a lineage which has been expanding as a cause of IPD in children and adults [19,30], and that became the single most important serotype among PNSP and ERP in the last three years of the study. This was accompanied by increases in serotypes including resistant isolates not represented in PCV13, such as serotypes 6C, 15A, 29, 33A, as well as non-typable isolates, each including $n>5$ PNSP or ERP during the entire study period (table 2).

The major limitation of this study is that we do not know if blood cultures were performed for all patients, and so we cannot exclude the possibility that some of the isolates attributed to NIPP were in fact reflecting cases of invasive disease. However, the distinct serotype distribution between IPD and NIPP and the similar distribution found in this study and among isolates causing NIPP in Denmark in a similar period [5], in spite of the different epidemiological contexts, strongly argues against a significant bias in our sample. Another possible confounder could be that a fraction of our isolates are reflecting colonization and not infection. Again we consider this unlikely. The fluids included are not present in healthy subjects (sputum and bronchial secretions) or are not obtained unless there is a strong suspicion of pneumonia (bronchoalveolar lavage). The participating laboratories used criteria to exclude low quality samples, which would be

more likely to reflect upper airway microbiota. Finally, adult colonization is known to be rare [31] and would be therefore unlikely to account for a significant fraction of the isolates. Taken together, these arguments support a role for the pneumococci analyzed in infection and not asymptomatic colonization. The decision to collect specimens for microbiological analysis was the responsibility of the attending physician that did not receive specific guidelines. We are not aware of significant changes in practice during the study period, although differences between the participating centers may exist. However, since these are expected to be minor and stable during the study period we do not feel these constitute a significant source of bias.

In this study, we found a different serotype distribution and dynamics in NIPP and IPD in the same population. This was highlighted by the fact that the potential coverage of the currently available pneumococcal vaccines with an adult indication is lower in NIPP than in IPD. The distinct dynamics of NIPP, the availability of PCV13 for adults together with the issues raised regarding the efficacy of PPV23 in the context of NIPP, and the fact that NIPP is a frequent cause of morbidity and mortality among adults, all underscore the relevance of considering the use of PCV13 in adults. However, the expected herd protection conferred by vaccinating children with PCV13 could reduce the benefits of direct adult vaccination. We documented here ongoing changes in the serotypes causing NIPP that are potentially due to long-term PCV7 use, but there is uncertainty regarding the ultimate reduction in vaccine serotypes one can expect from this effect, as well as regarding the kinetics of such a decline. Continued surveillance is essential to evaluate the changing potential benefits of direct adult vaccination.

Supporting Information

Figure S1 Proportion of isolates of each of the serotypes that together were responsible for half of non-invasive pneumococcal pneumonia isolates and half of invasive pneumococcal disease cases in adults in Portugal (2009–2011). Data from IPD were published previously [19].
(PDF)

Figure S2 Proportion of isolates expressing serotypes included in pneumococcal vaccines causing non-invasive pneumococcal pneumonia in adults in Portugal (1999–2003).
(PDF)

Figure S3 Proportion of isolates of each of the serotypes that together were responsible for half of non-invasive pneumococcal pneumonia isolates and half of invasive pneumococcal disease cases in adults in Portugal (2009–2011). Data from IPD were published previously (19).
(PDF)

Table S1 Serotype distribution of the 10 most common serotypes responsible for non-invasive pneumococcal pneumonia in adults in Portugal, stratified by age groups (1999–2011).
(PDF)

Table S2 Isolates expressing vaccine serotypes responsible for non-invasive pneumococcal pneumonia in adults in Portugal, stratified by age groups (2009–2011).
(PDF)

Table S3 Serotype distribution of the 10 overall most common serotypes in NIPP and in IPD (2009–2011).
(PDF)

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Author Contributions

Conceived and designed the experiments: JM MR. Performed the experiments: ANH JPL TPGSS. Analyzed the data: JM MR ANH. Contributed reagents/materials/analysis tools: TPGSS. Contributed to the writing of the manuscript: JM MR ANH.

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PLOS ONE

RESEARCH ARTICLE

Conjugate vaccine serotypes persist as major causes of non-invasive pneumococcal pneumonia in Portugal despite declines in serotypes 3 and 19A (2012-2015)

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Abstract

Non-invasive pneumococcal pneumonia (NIPP) is a frequent cause of morbidity and mortality worldwide. The 13-valent pneumococcal conjugate vaccine (PCV13) was included in the national immunization program of children living in Portugal in 2015. Until then, PCV7 (since late 2001) and PCV13 (since early 2010) were given through the private market. We determined the serotype distribution and antimicrobial susceptibility of isolates causing adult NIPP in 2012–2015 and compared the results with previously published data (2007–2011). There were 50 serotypes among the 1435 isolates. The most common were serotypes: 3 (14%), 11A (8%), 19F (6%), 23A (5%), 6C (5%), 19A (4%), 23B (4%), 9N (4%) and non-typable isolates (4%). When considering data since the availability of PCV13 for children in the private market, the proportion of PCV13 serotypes declined from 44.0% in 2010 to 29.7% in 2015 ($p < 0.001$), mainly due to early decreases in the proportions of serotypes 3 and 19A. In contrast, during the same period, PCV7 serotypes (11.9% in 2012–2015) and the serotypes exclusive of the 23-valent polysaccharide vaccine (26.0% in 2012–2015), remained relatively stable, while non-vaccine types increased from 27.0% in 2010 to 41.9% in 2015 ($p<0.001$). According to the Clinical and Laboratory Standards Institute (CLSI) breakpoints, penicillin non-susceptible and erythromycin resistant isolates accounted for 1% and 21.7%, respectively, of the isolates recovered in 2012–2015, with no significant changes seen since 2007. Comparison of NIPP serotypes with contemporary invasive disease serotypes identified associations of 19 serotypes with either disease presentation. The introduction of PCV13 in the national immunization program for children from 2015 onwards may lead to reductions in the proportion of NIPP due to vaccine serotypes but continued NIPP surveillance is essential due to a different serotype distribution from invasive disease.

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Introduction

Pneumococcal pneumonia is among the most frequent causes of death due to infection worldwide, particularly among young children and older adults [1]. Non-invasive pneumococcal pneumonia (NIPP) is three to ten times more frequent than bacteremic pneumonia [2], but studies evaluating NIPP are less abundant than those evaluating invasive pneumococcal disease (IPD).

After the introduction of pneumococcal conjugate vaccines (PCVs) for children, several studies reported a reduction of IPD in children [3,4]. Given that young children are the main reservoirs and transmitters of pneumococcus in the community and because the PCVs reduce pneumococcal colonization, several studies also reported reductions of IPD due to vaccine serotypes in the non-vaccinated population [5–8].

Despite the lower number of studies, there is also evidence of herd protection in adult NIPP [9–12]. One study from the Netherlands suggested that, based on the similarity of vaccine serotype trends between NIPP and IPD, their national IPD data could be used to extrapolate the trends of NIPP [13]. However, there are also reasons to question predictions of NIPP trends from IPD data in all settings. Perhaps the most significant is that serotype distribution and the proportion of disease that is due to vaccine serotypes differs geographically and between IPD and NIPP [14,15]. Moreover, vaccine serotypes are free to circulate in unvaccinated people so that, especially in countries where the PCVs are not included in national immunization programs, these can persist as causes of disease, both of NIPP and IPD.

In Portugal, PCVs were available only outside the national immunization program for pediatric use until mid-2015. The first PCV to become available was the 7-valent formulation (PCV7), in late-2001. Although the cost of vaccination was fully supported by the parents, the initially modest uptake of PCV7 increased steadily, reaching 75% in 2008 [16]. A 13-valent formulation (PCV13) replaced PCV7 in early-2010 but uptake declined, although it stayed above 60% [17]. In June 2015, PCV13 was included in the national immunization program to be given free of charge to all children born from January 2015 onwards, with a 2+1 schedule [17]. Besides children, sequential vaccination with PCV13 and the 23-valent pneumococcal polysaccharide vaccine (PPV23) is recommended since 2015 by the national health authorities, but only for specific risk groups [18]. In addition, two Portuguese medical societies (respiratory society and general practitioner society) have issued recommendations for the sequential vaccination with PCV13 and PPV23 of all immunocompetent adults $\geq$65 years [19,20]. Still, pneumococcal vaccine uptake in adults is generally believed to be low, with a study finding that <9% of all adults $\geq$65 years had received PPV23 [21,22].

In a previous study we analyzed the distribution of serotypes in a randomly selected sample of 100 isolates/year collected from adult NIPP between 1999 and 2011 [14]. In the present study we aimed to gain further insights regarding vaccine serotype trends in adult NIPP in the years that followed. We characterized isolates causing adult NIPP throughout Portugal from 2012 to 2015 for serotype distribution and antimicrobial susceptibility. We also wanted to compare the NIPP data with contemporary adult IPD data obtained by the same network.

Materials and methods

Ethics statement

The study was approved by the Institutional Review Board of the Centro Académico de Medicina de Lisboa. These were considered surveillance activities and were exempt from informed consent. All methods were performed in accordance with the relevant guidelines

and regulations. The data and isolates were de-identified so that these were irretrievably unlinked to an identifiable person.

Bacterial isolates

Isolates were provided by a laboratory-based surveillance system that includes 30 microbiology laboratories throughout Portugal. These were asked to submit all consecutively collected pneumococci causing infections to the central laboratory. Although the laboratories were contacted periodically to submit the isolates to the central laboratory, no audit was performed to ensure compliance, which may be variable in this type of study. The identification of all isolates as *Streptococcus pneumoniae* was confirmed by colony morphology and hemolysis on blood agar plates, optochin susceptibility and bile solubility.

The isolates included in this study were recovered from sputum, bronchial secretions or bronchoalveolar lavage of adult patients ($\geq$18 yrs) with a presumptive diagnosis of pneumonia between 2012 and 2015. Isolates were not included when pneumococci were simultaneously isolated from blood or another usually sterile product, and when other potential bacterial pathogens besides pneumococci were detected in the sample (such as *Haemophilus influenzae*, which was also frequently detected). Only one isolate from each patient in each year was considered.

Serotyping and antimicrobial susceptibility testing

Serotyping was performed by the standard capsular reaction test using the chessboard system and specific sera (Statens Serum Institut, Copenhagen, Denmark) [23]. Serotypes were classified into vaccine serotypes, i.e., those included in PCV7 (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F), in PCV13 (all PCV7 serotypes and the additional serotypes present only in PCV13, addPCV13: 1, 3, 5, 6A, 7F and 19A), in PPV23 (all PCV13 serotypes, except serotype 6A, and the additional serotypes present only in PPV23, addPPV23: 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F) and non-vaccine serotypes (NVT, including all other serotypes). Given the high frequency of spontaneous switching between serotypes 15B and 15C we have opted to group isolates with these serotypes into a single group. Due to difficulties in phenotypically distinguishing isolates of serotype 25A and serogroup 38 and of serogroup 29 and serotype 35B these were also grouped together into the 25A/38 and 29/35B groups. The isolates that were not typable with any of the complete set of sera were considered non-typable (NT).

Minimum inhibitory concentrations (MICs) for penicillin and cefotaxime were determined using Etest strips (Biomérieux, Marcy-L'Etoile, France). Unless otherwise stated, the results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) recommended breakpoints prior to 2008 [24], corresponding to the current breakpoints of oral penicillin V allowing the comparison with previously published data. According to these criteria, intermediate resistance to penicillin is defined as MIC 0.12–1.0 μg/ml and high-level resistance as MIC$\geq$2.0 μg/ml. Isolates that fell into either one of these classes were designated penicillin non-susceptible (PNSP). The interpretation according to the current CLSI guidelines was also performed [25]. According to these criteria, for non-meningitis cases, intermediate resistance to penicillin is defined as MIC between 2–8 μg/ml and high-level resistance as MIC>8 μg/ml. Susceptibility to cefotaxime was defined as MIC$\leq$1.0 μg/ml. The Kirby-Bauer disk diffusion assay was used to determine susceptibility to levofloxacin, erythromycin, clindamycin, chloramphenicol, trimethoprim/sulfamethoxazole, tetracycline, vancomycin and linezolid, according to the CLSI recommendations and interpretative criteria [25]. Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin, as previously described [14]. The MLS_B phenotype (resistance to macrolides, lincosamides and

streptogramin B) was defined as the simultaneous resistance to erythromycin and clindamycin, while the M phenotype (resistance to macrolides) was defined as non-susceptibility only to erythromycin.

Statistical analysis

Sample diversity was measured using Simpson's index of diversity (SID) and the respective 95% confidence intervals (CI95%) [26]. To compare two sets of partitions the Adjusted Wallace (AW) coefficients were calculated [26] using the online tool available at www.comparingpartitions.info. Differences were evaluated by the Fisher exact test with the false discovery rate (FDR) correction for multiple testing [27] or the Chi-squared test, and the Cochran-Armitage test was used for trends. A $p<0.05$ was considered significant for all tests.

Results

Serotype distribution

A total of 1435 isolates were collected from adults with non-invasive pneumococcal pneumonia: n = 368 in 2012, n = 319 in 2013, n = 311 in 2014 and n = 437 in 2015. Stratifying by age group, 339 isolates (23.6%) were recovered from patients 18–49 years old, 382 (26.6%) from patients 50–64 years old and 714 (49.8%) from patients ≥65 years old. Most of the isolates were recovered from sputum (n = 787, 54.8%), 531 (37.0%) were recovered from bronchial secretions and 117 (8.2%) were recovered from bronchoalveolar lavage fluid. A total of 50 different serotypes were detected. The most frequent serotypes, which accounted for 52% of the isolates, were serotypes 3 (n = 196, 13.7%), 11A (n = 120, 8.4%), 19F (n = 85, 5.9%), 23A (n = 67, 4.7%), 6C (n = 64, 4.5%), 19A (n = 58, 4.0%), 23B (n = 56, 3.9%), 9N (n = 52, 3.6%) and NT isolates (n = 50, 3.5%).

The S1 Fig represent the number of isolates expressing serotypes included in PCVs, the addPPV23, and the number of isolates expressing NVTs, respectively, stratified by age group. Serotype diversity was high—SID = 0.952, CI95%: 0.948–0.956 –with no difference between SIDs of different years. No individual serotype (n>15 isolates) showed differences in age distribution, statistically supported after FDR correction.

Fig 1 shows the proportion of potentially vaccine preventable NIPP during the study period and, for comparison purposes, also the previously published data from 2007–2011, since these years represent the late post-PCV7 period (2007–2009) and the first two years of PCV13 use in children (2010–2011) [14]. Considering the evolution during the current study period only (2012–2015), there was a decline in the proportion of NIPP caused by PCV13 serotypes, from 34.5% in 2012 to 29.7% in 2015, but this was not statistically supported (p = 0.090). This decline was associated with slight and non-significant decreases in both the proportion of NIPP caused by PCV7 serotypes (from 13.6% to 11.0%, p = 0.177) and addPCV13 (from 20.9% to 18.8%, p = 0.377). In contrast, there was a non-significant increase in the proportion of NIPP caused by addPPV23 (from 24.7% in 2012 to 28.4% in 2015, p = 0.325), while the proportion of NIPP caused by NVTs remained relatively stable from 2012 to 2015 (40.8% vs 41.9%, respectively, p = 0.460).

We then evaluated possible serotype trends since 2010 when PCV13 started being used in children through the private market. The overall proportion of PCV13 serotypes declined from 44.0% in 2010 to 29.7% in 2015 ($p<0.001$), while that of addPCV13 decreased from 36.0% in 2010 to 18.8% in 2015 ($p<0.001$). This was accompanied by an increase of NVTs from 27.0% in 2010 to 41.9% in 2015 (p = 0.002). The PCV7 and addPPV23 serotypes remained relatively stable.

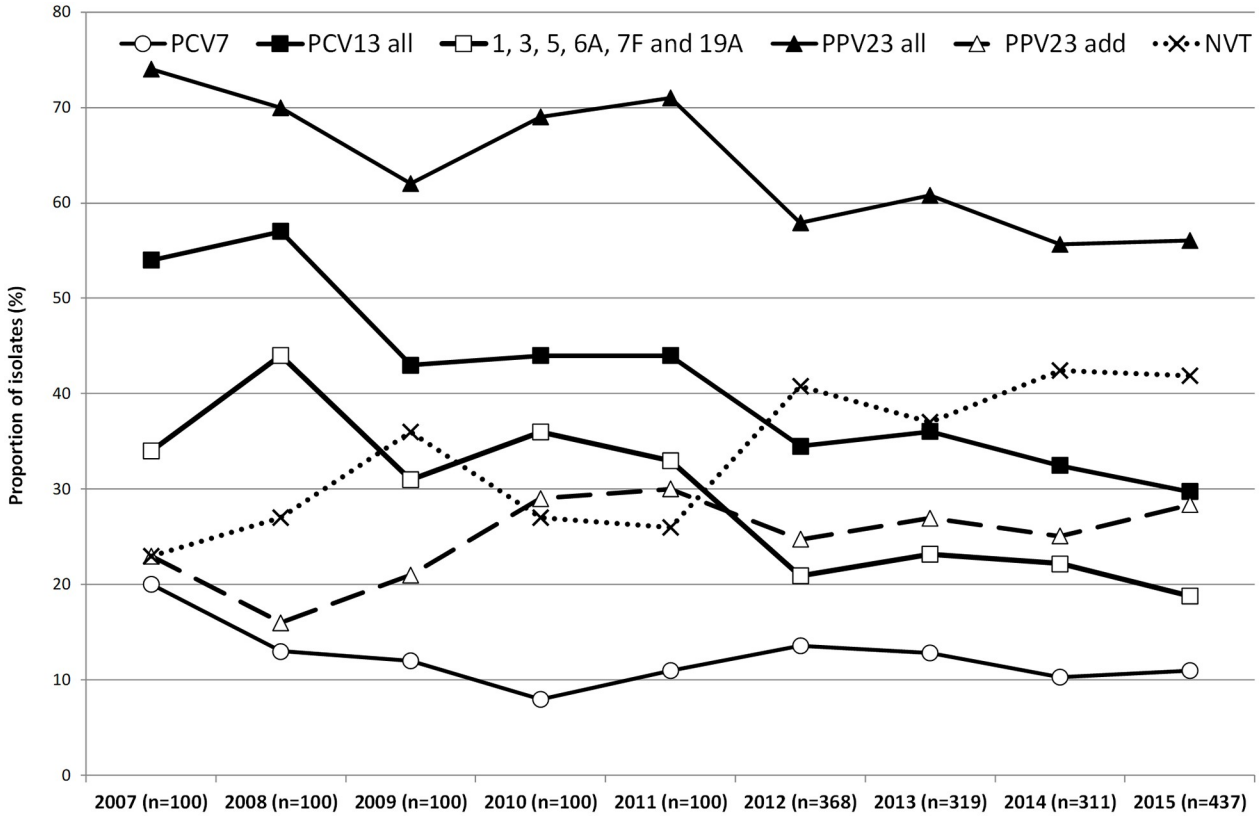

Fig 1. Proportion of isolates expressing serotypes included in each of the pneumococcal vaccines causing non-invasive pneumococcal pneumonia in adult patients (≥18 years) in Portugal, 2007–2015. The data up to 2011 were presented previously [14].

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Table 1 shows the evolution of the individual serotypes causing NIPP in adults during the current study period (2012–2015). Only serotype 25A/38 significantly changed its proportion after FDR correction (from 1.9% in 2012 to 0.0% in 2015, p = 0.001). When considering the evolution of individual serotypes since 2007 (Table 1 and S1 Table), only four serotypes significantly changed their proportion after FDR correction (S2 Fig), which were serotypes 3 (declined from 22.0% in 2007 to 12.1% in 2015, p<0.001), 19A (declined from 6.0% in 2007 to 3.2% in 2015, p = 0.003), 7F (declined from 3.0% in 2007 to 1.1% in 2015, p = 0.004) and 35F (increased from 0% in 2007 to 3.2% in 2015, p = 0.003). The declines in proportion of serotypes 3 and 19A showed important yearly fluctuations and these were also found for several other serotypes (Table 1 and S2 Table).

When analyzing the evolution of individual vaccine serotypes and of vaccine serotype groups in 2012–2015 stratified by age group (Table 2), there were no significant changes after FDR correction. When considering data since 2007, only for serotype 3 and for adults aged ≥65 years old did the change remain statistically supported after FDR correction (serotype 3 declined from 27.5% in 2007 to 11.1% in 2015, p<0.001).

Antimicrobial susceptibility

Susceptibility to the tested antimicrobials between 2012 and 2015 stratified by the age groups considered is summarized in Table 3. When considering all isolates, a total of n = 258 isolates (18.0%) were classified as PNSP of which n = 229 (88.8%) expressed low-level resistance and n = 29 (11.2%), high-level resistance. According to the current CLSI guidelines for parental

Table 1. Serotypes of the isolates responsible for non-invasive pneumococcal pneumonia in adult patients (≥18 years), 2012–2015.

Serotype	No. of isolates (%)				CA[a]
	2012	2013	2014	2015	2012–2015
PCV13					
1	2 (0.5)	0 (0)	0 (0)	1 (0.2)	0.399
3	48 (13.0)	54 (16.9)	41 (13.2)	53 (12.1)	0.408
4	1 (0.3)	1 (0.3)	2 (0.6)	3 (0.7)	0.329
5	0 (0)	0 (0)	0 (0)	0 (0)	-
6A	5 (1.4)	7 (2.2)	6 (1.9)	9 (2.1)	0.547
6B	7 (1.9)	4 (1.3)	4 (1.3)	6 (1.4)	0.578
7F	5 (1.4)	4 (1.3)	4 (1.3)	5 (1.1)	0.800
9V	0 (0)	5 (1.6)	0 (0)	0 (0)	0.275
14	12 (3.3)	6 (1.9)	6 (1.9)	10 (2.3)	0.426
18C	4 (1.1)	1 (0.3)	1 (0.3)	1 (0.2)	0.106
19A	17 (4.6)	9 (2.8)	18 (5.8)	14 (3.2)	0.645
19F	22 (6.0)	22 (6.9)	15 (4.8)	26 (5.9)	0.746
23F	4 (1.1)	2 (0.6)	4 (1.3)	2 (0.5)	0.483
PPV23 only					
8	7 (1.9)	10 (3.1)	6 (1.9)	16 (3.7)	0.222
9N	11 (3.0)	13 (4.1)	16 (5.1)	12 (2.7)	0.942
10A	11 (3.0)	6 (1.9)	5 (1.6)	10 (2.3)	0.519
11A	29 (7.9)	29 (9.1)	22 (7.1)	40 (9.2)	0.703
12F	0 (0)	0 (0)	0 (0)	0 (0)	-
15B/C	6 (1.6)	11 (3.4)	7 (2.3)	10 (2.3)	0.807
17F	8 (2.2)	4 (1.3)	8 (2.6)	4 (0.9)	0.319
20	5 (1.4)	5 (1.6)	6 (1.9)	8 (1.8)	0.557
22F	14 (3.8)	8 (2.5)	6 (1.9)	21 (4.8)	0.448
33F	0 (0)	0 (0)	2 (0.6)	3 (0.7)	0.048
NVT[b]					
6C	19 (5.2)	16 (5.0)	7 (2.3)	22 (5.0)	0.627
23A	24 (6.5)	12 (3.8)	14 (4.5)	17 (3.9)	0.130
23B	15 (4.1)	8 (2.5)	15 (4.8)	18 (4.1)	0.631
NT	9 (2.4)	8 (2.5)	16 (5.1)	17 (3.9)	0.123
15A	10 (2.7)	9 (2.8)	8 (2.6)	16 (3.7)	0.465
31	15 (4.1)	7 (2.2)	14 (4.5)	12 (2.7)	0.587
16F	7 (1.9)	10 (3.1)	3 (1.0)	20 (4.6)	0.070
29/35B	12 (3.3)	6 (1.9)	6 (1.9)	10 (2.3)	0.426
35F	5 (1.4)	3 (0.9)	6 (1.9)	14 (3.2)	0.033
34	3 (0.8)	8 (2.5)	5 (1.6)	8 (1.8)	0.445
21	3 (0.8)	6 (1.9)	7 (2.3)	8 (1.8)	0.265
24F	4 (1.1)	2 (0.6)	6 (1.9)	10 (2.3)	0.082
33A	6 (1.6)	1 (0.3)	5 (1.6)	0 (0)	0.052
25A/38	7 (1.9)	2 (0.6)	0 (0)	0 (0)	**0.001**
35A	2 (0.5)	3 (0.9)	4 (1.3)	2 (0.5)	0.946
7C	2 (0.5)	2 (0.6)	6 (1.9)	1 (0.2)	0.946
13	2 (0.5)	3 (0.9)	1 (0.3)	1 (0.2)	0.333
37	1 (0.3)	4 (1.3)	1 (0.3)	3 (0.7)	0.802
Others[c]	4 (1.1)	8 (2.5)	8 (2.6)	4 (0.9)	-

(*Continued*)

Table 1. (Continued)

Serotype	No. of isolates (%)				CA[a]
	2012	2013	2014	2015	2012–2015
Total	368	319	311	437	-

[a]CA, Cochran Armitage test of trend. In bold is the only serotype with significant p-value ($p < 0.05$) after FDR correction.

[b]NVT, non-vaccine serotypes, i.e., serotypes not included in any of the currently available pneumococcal vaccines.

[c]Only serotypes detected in ≥3 isolates in at least one year are shown; the remaining are grouped together under "Others."

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penicillin in non-meningitis cases [25], only n = 15 isolates (1.0%) would have been considered PNSP, with only 2 of these expressing high-level resistance. A total of n = 311 isolates (21.7%) were classified as erythromycin resistant pneumococci (ERP). Of these, n = 246 isolates (79.1%) expressed the MLS_B phenotype, while the remaining (n = 65, 20.9%) presented the M phenotype. A total of 12.3% (n = 176) of the isolates were simultaneously non-susceptible to penicillin and resistant to erythromycin (EPNSP).

There were no significant variations in antimicrobial resistance during the current study period (2012–2015), nor were there significant changes in antimicrobial resistance when considering NIPP from 2007 [14]. Although with moderate overall AW values [the AW for serotype to PNSP was 0.441 (CI95%: 0.386–0.496) and the AW for serotype to ERP was 0.443 (CI95%: 0362–0.524)], there was an association between certain serotypes and antimicrobial

Table 2. Number of isolates responsible for non-invasive pneumococcal pneumonia in adult patients (≥18 years), according to vaccine serotype groups and age groups, 2012–2015.

	Serotype Groups[b]	No. isolates (%)			2015	CA[a]
		2012	2013	2014		
18–49 years	PCV7	12 (12.9)	11 (16.4)	8 (10.3)	16 (15.8)	0.782
	1, 5 and 7F	1 (1.1)	1 (1.5)	2 (2.6)	1 (1.0)	0.926
	3, 6A and 19A	19 (20.4)	11 (16.4)	10 (12.8)	20 (19.8)	0.800
	PCV13	32 (34.4)	23 (34.3)	20 (25.6)	37 (36.6)	0.983
	addPPV23	25 (26.9)	15 (22.4)	28 (35.9)	30 (29.7)	0.417
	NVTs	36 (38.7)	29 (43.3)	30 (38.5)	34 (33.7)	0.385
50–64 years	PCV7	12 (14.1)	7 (8.4)	7 (7.4)	8 (6.7)	0.328
	1, 5 and 7F	3 (3.5)	0 (0)	1 (1.1)	1 (0.8)	0.656
	3, 6A and 19A	15 (17.6)	23 (27.7)	23 (24.2)	19 (16.0)	0.095
	PCV13	30 (35.3)	30 (36.1)	31 (32.6)	28 (23.5)	0.026
	PPV23 add	45 (18.8)	50 (27.7)	53 (24.2)	65 (32.8)	0.082
	NVTs	39 (45.9)	30 (36.1)	41 (24.2)	52 (32.8)	0.531
≥65 years	PCV7	26 (13.7)	23 (13.6)	17 (12.3)	24 (1.1)	0.381
	1, 5 and 7F	3 (1.6)	3 (1.8)	1 (0.7)	4 (1.8)	0.976
	3, 6A and 19A	36 (18.9)	36 (21.3)	32 (23.2)	37 (17.1)	0.665
	PCV13	65 (34.2)	62 (36.7)	50 (36.2)	65 (30.0)	0.332
	PPV23 add	50 (26.3)	48 (28.4)	27 (19.6)	55 (25.3)	0.078
	NVTs	75 (39.5)	59 (34.9)	61 (44.2)	97 (44.7)	0.125

[a] CA, Cochran Armitage test of trend.

[b] PCV7, serotypes included in the 7-valent pneumococcal conjugate vaccine. PCV13, serotypes included in the 13-valent pneumococcal conjugate vaccine. addPPV23, the additional 11 serotypes present in the 23-valent pneumococcal polysaccharide vaccine but absent from PCV13. NVTs, serotypes not included in any of the currently available pneumococcal vaccines.

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Table 3. Antimicrobial resistance of the isolates responsible for non-invasive pneumococcal pneumonia in adult patients (≥18 years) in Portugal, 2012–2015.

	No. resistant isolates (%)[a]		
	18–49 years (n = 339)	50–64 years (n = 382)	≥65 years (n = 714)
PEN[b]	57 (16.8)	70 (18.3)	131 (18.3)
MIC90	0.19	0.19	0.38
MIC50	0.012	0.012	0.012
CTX	7 (2.1)	7 (1.8)	4 (0.6)
MIC90	0.25	0.25	0.38
MIC50	0.015	0.016	0.016
LEV	2 (0.6)	3 (0.8)	16 (2.2)
ERY	73 (21.5)	70 (18.3)	168 (23.5)
CLI	56 (16.5)	58 (15.2)	136 (19.0)
CHL	7 (2.1)	7 (1.8)	4 (0.6)
SXT	57 (16.8)	66 (17.3)	104 (14.6)
TET	59 (17.4)	55 (14.4)	115 (16.1)

[a]PEN, penicillin; CTX, cefotaxime; LEV, levofloxacin; ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline. All isolates were susceptible to vancomycin and linezolid.
[b]Non-susceptibitily to penicillin was determined using the CLSI breakpoints prior to 2008 [24].

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resistance (S1 Fig). The serotypes that were positively associated with PNSP after FDR correction were serotypes 6C, 14, 15A, 19F, 19A and 23F. Among these, serotypes 19F (15.5%), 14 (12.4%), 6C (12.0%) and 19A (11.6%) accounted for half of all PNSP. The serotypes which were positively associated with ERP after FDR correction were serotypes 6B, 6C, 14, 15A, 19F, 19A, 33A and 35A, of which serotypes 19F (20.3%), 19A (10.6%), 6C (9.6%) and 15A (8.0%) accounted for half of all ERP. The PCV7, PCV13 and PPV23 serotypes accounted for 33.7%, 47.3% and 53.5% of PNSP, respectively, and 33.4%, 49.5% and 54.3% of ERP, respectively.

Discussion

The present study documented a decline of PCV13 serotypes in adult NIPP in the post-PCV13 period. This occurred mostly in 2011–2012 but continued, albeit more moderately, in recent years, from 44.0% in 2010 to 29.7% in 2015. It was also noted that during 2007–2015 there were several important yearly fluctuations in the proportion of individual serotypes, both among PCV13 and non-PCV13 serotypes (Table 1 and S1 Table). This suggests that variations of the PCV13 serotypes in the post-PCV13 period in adult NIPP could be the result of, not only the herd protection conferred by childhood vaccination with PCV13, but also of temporal trends, which had been documented in adult NIPP in Portugal previously [14].

The evolution of PCV13 serotypes in adult NIPP from 2010 onwards (when PCV13 was being used for children vaccination in the private market) was different from the one previously found for adult IPD in Portugal in a similar period [6]. While in NIPP the sharpest decrease in addPCV13 serotypes in the post-PCV13 period occurred from 2011 to 2012, in IPD this occurred only from 2012 to 2013. Although the decrease of addPCV13 serotypes in adult NIPP may have been also influenced by temporal trends, the sustained lower values found from 2013 onwards suggest an important contribution of herd protection resulting from PCV13 childhood vaccination.

Serotypes 3 and 19A had a major influence in the decrease of PCV13 serotypes in adult NIPP in the post-PCV13 period (2010–2015), although these changes were also most

significant in the first years (Table 1 and S1 Table). Similarly, in adult IPD two serotypes accounted for most of the decline in the prevalence of PCV13 serotypes in the post-PCV13 period, but in this case these were serotypes 7F and 19A [6]. In contrast with the declines of serotypes 3 and 19A in adult NIPP, the decreases of serotypes 7F and 19A in adult IPD were more pronounced and sustained.

Serotype 3 has been the dominant serotype in adult NIPP and IPD in Portugal, both before and after the introduction of PCV13 for children [6,14]. The decline in serotype 3 in NIPP is surprising because this serotype did not show major changes in adult IPD in the post-PCV13 period in Portugal [6] nor in other countries [7,8,28–30]. However, reductions in incidence of serotype 3 NIPP was reported in other studies, including a study from England [10]. The reduced efficacy of PCV13 in preventing pediatric complicated pneumonias caused by serotype 3 [17] and the use of PCV13 outside of the national immunization program with somewhat modest uptake, raise the possibility of continued circulation of this serotype in carriage, potentially explaining its persistence in disease. Since serotype 3 is heterogeneous in its invasive disease potential, meaning that there are different clones expressing this serotype that differ in their capacity to cause invasive disease [31], it is possible that more invasive clones of serotype 3 have increased post-PCV use for reasons that remain unknown. In adult IPD, there was an expansion of the multilocus sequence type clonal complex CC180 among isolates expressing serotype 3 [32], prior to the use of PCV13 in children, but no information is available in the post-vaccine period.

Serotype 19A emerged in Portugal in the late post-PCV7 period, to become one of the most important serotypes in both adult NIPP [14] and IPD [5,6,33]. A decrease of serotype 19A in adult IPD and in NIPP in the post-PCV13 period was documented not only for Portugal but for other countries [7,8,10,11,30]. Given the compelling evidence of herd protection in adult IPD resulting from PCV13 use in children in serotype 19A, the lack of a more significant reduction of serotype 19A in adult NIPP in the post-PCV13 period could be due to a particular propensity of this serotype to cause NIPP. A clearer picture of the impact of PCV13 use in children in reducing the importance of serotypes 3 and 19A in adult NIPP may only be provided by further studies following the epidemiology of adult NIPP after the inclusion of PCV13 in the national immunization plan.

A decrease in serotype 7F was also detected but its contribution to the reduction of PCV13 serotypes in NIPP was minor since this serotype was an uncommon cause of NIPP in the pre-PCV13 period.

Contrasting with the declining trend of PCV13 serotypes, no significant trend was seen for PCV7 serotypes in adult NIPP and this was mostly due to the persistence of serotype 19F, which occurred in 49% of the isolates expressing a PCV7 serotype in 2012–2015. Despite being targeted by all PCVs available to date, serotype 19F remained common in nasopharyngeal carriage of children in Portugal in the late post-PCV7 period [34] and in the post-PCV13 period [35], including among vaccinated children. The inability of the PCVs to eliminate this serotype from carriage in children, at least in a non-universal coverage scenario, together with its likely intrinsic propensity to cause NIPP rather than IPD [14] as was also shown here, may have contributed to why this serotype remained the third most frequent cause of adult NIPP in the post-PCV13 period in Portugal.

The decrease of PCV13 serotypes in the post-PCV13 period was accompanied by an increase in the proportion of NVTs, while the addPPV23 serotypes remained relatively stable. However, among the NVTs, only one serotype was clearly emerging (serotype 35F) and only in the last year of the study period. Most of the remaining increase in NVTs was based in increases in the proportion of serotypes 16F, 24F and NTs (Table 1 and S1 Table), which were not significant if considered independently. This contrasts with results from adult IPD, in

which there were several non-PCV13 emerging serotypes (serotypes 8, 22F, 20 and 15A), most of them included in PPV23 [6]. These differences are not surprising, since isolates responsible for adult NIPP and adult IPD are known to have different serotype distributions [14,15].

When comparing the serotype distribution of isolates causing adult NIPP in 2012–2014 with the serotype distribution of isolates causing adult IPD in the same period (S2 Table), serotypes 11A, 19F, 23A, 23B, 31, NT, 17F, 6A, 21 and 37 (ranked by their frequency in NIPP) were significantly associated with NIPP, while serotypes 8, 19A, 22F, 14, 7F, 20, 1, 4 and 12B (ranked by their frequency in IPD) were significantly associated with IPD. Most of these associations had been already recognized in the pre-PCV13 period [14], while the new associations in adult IPD reflect mainly the emerging serotypes in the post-PCV13 period.

While antimicrobial resistance declined in adult IPD in the post-PCV13 period, no decline was found for adult NIPP in this study. In NIPP, the small decrease in proportion of the mostly antimicrobial resistant serotype 19A isolates, was balanced by an increase of NT isolates, which were also associated with antimicrobial resistance. NTs were found to be frequent colonizers of the nasopharynx of children in the post-PCV13 period [34] and were more frequently found in NIPP than in IPD (S2 Table). The stability of PCV7 serotypes in the post-PCV13 period also helped maintaining antimicrobial resistance rates in adult NIPP [14].

The study presented has the limitations discussed previously [14]. These include the possibility that some of the isolates we identified as being responsible for NIPP were in fact causing bacteremic pneumonia or reflected colonization and not disease. Despite the general recommendation that both blood and respiratory tract samples should be collected for the etiologic diagnosis of pneumonia, we cannot guarantee that this was done in all cases. However, we consider these to account, at most, for a small fraction of the isolates and therefore not to introduce a significant bias. Moreover, the distinct serotype distribution found in this study for IPD and NIPP, strongly argues against this possibility. Since our study is laboratory-based, it was not designed to collect information important to assess the severity of the infections caused by the different serotypes (e.g. hospitalization, ICU admission, 30-day mortality). However, this does not compromise our approach of comparing the serotype distribution of IPD and NIPP cases. Our temporal analyses were based on previously published data reporting the characteristics of a random sample of 100 isolates per year [14]. Since not all available isolates before 2012 were characterized, it is possible that some of the changes in the serotype distribution occurring from 2011 to 2012 are due to this sampling process.

In this study it was found that the overall proportion of PCV13 serotypes decreased only moderately in adult NIPP in the post-PCV13 period. In 2015, 30% of NIPP was due to PCV13 serotypes and 28% was due to the addPPV23 serotypes, highlighting the potential role of vaccination in disease prevention. However, the inclusion of PCV13 in the national immunization program for children in 2015 and the anticipated declines in at least some of the PCV13 serotypes due to herd effect, raise important issues regarding the cost-effectiveness of a universal adult vaccination program. However, because the magnitude and timeframe of this herd effect remains poorly defined, particularly in NIPP, further surveillance is essential to document future trends in pneumococcal serotype prevalence in adult NIPP, as these seem to differ from adult IPD.

Supporting information

S1 Fig. Number of isolates expressing each serotype causing non-invasive pneumococcal pneumonia in adult patients (≥18 yrs), Portugal, 2012–2015. The number of isolates expressing each serotype in each of the age groups considered is indicated. Isolates recovered from patients 18–49 years are indicated by black triangles. Isolates recovered from patients

50–64 years are indicated by open squares. Isolates recovered from patients ≥ 65years are indicated by open circles. Isolates presenting both erythromycin resistance and penicillin non-susceptibility (EPNSP) are represented by closed black bars. Penicillin non-susceptible isolates (PNSP) are indicated by dark hatched bars. Erythromycin resistant pneumococci (ERP) are indicated by light hatched bars. Isolates susceptible to both penicillin and erythromycin are represented by white open bars. **Panel A—Serotypes included in conjugate vaccines**. The serotypes included in the seven-valent conjugate vaccine (PCV7) and in the 13-valent conjugate vaccine (PCV13) are indicated by the arrows. NVT, non-vaccine serotypes; addPPV23, the additional serotypes included in the 23-valent polysaccharide vaccine but not included in PCV13. **Panel B—Additional serotypes included in the 23-valent polysaccharide vaccine but not included in the 13-valent conjugate vaccine**. Out of the 11 addPPV23 serotypes only serotype 2 was not found in our collection. **Panel C—Serotypes not included in any pneumococcal vaccine** NT, non-typable. Isolates expressing serotypes 25A and 38 and serotypes 29 and 35B could not be distinguished phenotypically and are represented together. Only serotypes including n>3 isolates are discriminated, all remaining serotypes are grouped together under the "Others" category grouping isolates of serotypes: 10B, 12B, 17A, 18A (n = 3 each); 10F, 11F, 11B and 47F (n = 2 each) and 28A, 35C, 36 and 42 (n = 1 each).
(PDF)

S2 Fig. Isolates expressing serotypes that changed in proportion after FDR correction causing non-invasive pneumococcal pneumonia in adult patients (≥18 years) in Portugal, 2007–2015. The data up to 2011 were presented previously [14].
(PDF)

S1 Table. Serotypes of the isolates responsible for non-invasive pneumococcal pneumonia in adult patients (≥18 years), 2007–2011. These data were presented previously [14].
(PDF)

S2 Table. Serotype distribution of the isolates causing non-invasive pneumococcal pneumonia and invasive pneumococcal disease in adults in Portugal (2012–2014).
(PDF)

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